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(54) Title: METHOD FOR EXPRESSION OF SMALL RNA MOLECULES WITHIN A CELL

(57) Abstract: The invention provides methods and compositions for the expression of small RNA molecules within a cell using a lentiviral vector. The methods can be used to express doubles stranded RNA complexes. Small interfering RNA (siRNA) can be expressed using the methods of the invention within a cell, which are capable of down regulating the expression of a target gene through RNA interference. A variety of cells can be treated according to the methods of the invention including embryos, embryogenic stem cells, allowing for the generation of transgenic animals or animals constituted partly by the transduced cells that have a specific gene or a group of genes down regulated.

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METHOD FOR EXPRESSION OF SMALL RNA MOLECULES WITHIN A CELL

Government Support

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Background of the Invention

Field of the Invention

[0002] The present invention relates generally to methods for altering gene expression in a cell or an animal using viral constructs engineered to deliver an RNA molecule. In a more specific aspect, a viral construct is used to deliver double-stranded RNA molecules that can be used to down-regulate or modulate gene expression.

Description of the Related Art

[0003] RNA interference (RNAi) or silencing is a recently discovered phenomenon (A. Fire *et al., Nature* 391, 806 (1998); C.E. Rocheleau *et al. Cell* **90**, 707 (1997)). Small interfering RNAs ("siRNAs") are double-stranded RNA molecules that inhibit the expression of a gene with which they share homology. siRNAs have been used as a tool to down regulate the expression of specific genes in a variety of cultured cells as well as in invertebrate animals. A number of such approaches have been reviewed recently (P.D. Zamore *Science* **296**, 1265 (2002)); however, such approaches have limitations. For example, no technique prior to the invention described herein allows for the generation of transgenic mammal having a specific gene down regulated through RNA interference. Similarly, there is a need for more robust methods for the introduction of small RNA molecules with regulatory function. The invention provided herein addresses these and other limitations in the field of RNA mediated gene regulation.

Summary of the Invention

[0004] The invention relates generally to methods to express within a cell an RNA molecule or molecules. These methods can be used with a wide variety of cell types.

RNA molecules can be expressed within a cell for a variety of purposes. For example, and without limitation, RNA molecules can serve as markers within a cell, can act as antisense oligonucleotides or ribozymes for regulating gene expression, and can serve to down regulate genes through RNA interference.

[0005] In one aspect, the invention provides retroviral constructs for the expression of an RNA molecule or molecules within a cell. The constructs preferably comprise a nucleic acid having the R and U5 sequences from a 5' lentiviral long terminal repeat (LTR), a self-inactivating lentiviral 3' LTR, and a RNA Polymerase III (pol III) promoter. The retroviral constructs preferably comprise an RNA coding region operably linked to the RNA Polymerase III promoter. The RNA coding region preferably comprises a DNA sequence that can serve as a template for the expression of a desired RNA molecule.

[0006] The RNA coding region can be immediately followed by a pol III terminator sequence which directs the accurate and efficient termination of RNA synthesis by pol III. The pol III terminator sequences generally comprise 4 or more consecutive T residues. In a preferred embodiment, a cluster of 5 consecutive Ts is used as the terminator by which pol III transcription is stopped at second or third T of the DNA template. As a result, only 2 to 3 U residues are added to the 3' end of the RNA that is synthesized from the RNA coding region.

[0007] A variety of pol III promoters can be used with the invention, including for example, the promoter fragments derived from H1 RNA genes or U6 sn RNA genes of human or mouse origin or from any other species. In addition, pol III promoters can be modified/engineered to incorporate other desirable properties such as to be inducible by small chemical molecules either ubiquitously or in a tissue-specific manner, for example, one activated with tetracycline or IPTG (lacI system).

[0008] The pol III promoter, RNA template region and pol III terminator together may comprise an "RNA cassette" or "RNA expression cassette." If the RNA is a small inhibitory RNA (siRNA), the expression cassette may be termed an "siRNA expression cassette."

[0009] In one embodiment, the RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region. Such an RNA molecule when expressed desirably forms a "hairpin" structure. The loop region is generally between about 2 and about 10 nucleotides in length. In a preferred

embodiment, the loop region is from about 6 and about 9 nucleotides in length. In one such embodiment of the invention, the sense region and the antisense region are between about 15 and about 30 nucleotides in length.

[0010] In one embodiment, the RNA coding region is operably linked downstream to an RNA Polymerase III promoter such that the RNA coding sequence can be precisely expressed without any extra non-coding nucleotides present at 5' end. In this way an RNA sequence can be expressed that is identical to a target sequence at the 5' end. The synthesis of the RNA coding region is ended at the terminator site. In one preferred embodiment the terminator consists of five consecutive T residues.

[0011] In another aspect of the invention, the retroviral vector can comprise multiple RNA coding regions. In one embodiment, the retroviral construct comprises a first RNA pol III promoter, a first coding region encoding a first RNA molecule operably linked to the first RNA pol III promoter, a second RNA pol III promoter and a second RNA coding region operably linked to the second RNA pol III promoter. Preferably, the second RNA coding region encodes an RNA molecule that is substantially complementary to the RNA molecule encoded by the first RNA coding region, such that the two RNA molecules can form a double-stranded structure when expressed. The methods of invention also include multiple RNA coding regions that encode hairpin-like self-complementary RNA molecules or other non-hairpin molecules.

[0012] In yet another embodiment of the invention, the retroviral construct comprises a first RNA pol III promoter operably linked to a first RNA coding region, and a second RNA pol III promoter operably linked to the same first RNA coding region in the opposite direction, such that expression of the RNA coding region from the first RNA pol III promoter results in a synthesis of a first RNA molecule as the sense strand and expression of the RNA coding region from the second RNA pol III promoter results in synthesis of a second RNA molecule as an antisense strand that is substantially complementary to the first RNA molecule. In one such embodiment, both RNA Polymerase III promoters are separated from the RNA coding region by termination sequences, preferably termination sequences having five consecutive T residues.

[0013] According to one embodiment of the invention, the 5' LTR sequences in the retroviral construct are derived from HIV. The retroviral construct may also comprise a

woodchuck hepatitis virus enhancer element sequence and/or a tRNA amber suppressor sequence.

[0014] In another embodiment of the invention, the self-inactivating 3' LTR is a U3 element with a deletion of its enhancer sequence. In yet another embodiment, the self-inactivating 3' LTR is a modified HIV 3' LTR.

[0015] The recombinant retroviral construct can be pseudotyped, for example with the vesicular stomatitits virus envelope glycoprotein.

[0016] In another aspect of the invention, expression of the RNA coding region results in the down regulation of a target gene. Preferably the target gene comprises a sequence that is at least about 90% identical with the RNA coding region, more preferably at least about 95% identical, and even more preferably at least about 99% identical.

[0017] According to a further aspect of the invention, the viral construct also comprises a nucleotide sequence encoding a gene of interest. The gene of interest is preferably operably linked to a Polymerase II promoter. Such a construct also can contain, for example, an enhancer sequence operably linked with the Polymerase II promoter.

[0018] A variety of Polymerase II promoters can be used with the invention, including for example, the CMV promoter. The RNA Polymerase II promoter that is chosen can be a ubiquitous promoter, capable of driving expression in most tissues, for example, the human Ubiquitin-C promoter, CMV β-actin promoter or PGK promoter. In other embodiments the RNA Polymerase II promoter is a tissue-specific promoter.

[0019] In one embodiment, the gene of interest is a marker or reporter gene, that can be used to verify that the vector was successfully transfected or transduced and its sequences expressed. In one such embodiment, the gene of interest is a fluorescent reporter gene, for example, the Green Fluorescent Protein. In yet another embodiment, the gene of interest is a drug resistant gene which can be used to select the cells that are successfully transduced. For example, the drug resistant gene can be the zeocin resistant gene (zeo). The gene of interest also can be a hybrid of a drug resistant gene and a fluorescent reporter gene, such as a zeo/gfp fusion. In another embodiment, the gene of interest encodes a protein factor that can regulate the transcription activity of inducible pol III promoters. In one of such embodiment, the gene of interest is tetR (repressor for tet operon) which regulates tetracycline responsive pol III promoters.

[0020] It is another aspect of the invention to provide methods for expressing an RNA molecule or molecules within a cell. According to the invention, a packaging cell line is transfected with a retroviral construct of the invention, recombinant retroviral particles are recovered from the packaging cell line; and a target cell is infected with the recombinant retrovirus particles.

[0021] In one embodiment of the invention, the target cell is an embryonic cell. An embryonic cell may be, for example, a single cell embryo or embryonic cells from within an early-stage embryo. In another embodiment of the invention, the target cell is an embryogenic stem cell. When the target cell is an embryonic cell, in one embodiment the embryonic cell is infected by injecting the recombinant retrovirus between the zona pellucida and the cell membrane of the embryonic cell. In another embodiment, the embryonic cell is infected by removing the zona pellucida and incubating the cell in solution containing the recombinant retrovirus. In such an embodiment, the zona pellucida can be removed, for example, by enzymatic digestion.

[0022] When the target cell is an embryonic cell or an embryogenic stem cell, the cell may be transplanted in a pseudopregnant female to generate a transgenic animal.

[0023] The methods of the invention also can be used with a variety of primary ex vivo normal or diseased cells or cells adapted in various tissue culture conditions from human, mouse and other vertebrates, including, without limitation, stem or precursor cells for the hematopoietic system, central nerve system cells, cells with regenerative capacities from a variety of other tissues and organs, dendritic cells and other developing and mature myeloid and lymphoid cells, and cancer cells derived from different cell lineages.

[0024] In a particular embodiment, the target cell is an embryonic cell of a bird within an egg. The embryonic cell of a bird is preferably infected by contacting the embryonic blastodisc of the bird egg with retroviral particles.

[0025] In yet another embodiment, the target cell is a fish egg. The fish egg is preferably infected by delivering the retroviral particles to the space between the chorion and the cell membrane of the fish egg.

Brief Description of the Drawings

[0026] Figure 1A shows a schematic diagram of a retroviral vector carrying an expression cassette for RNA expression, termed "RNA cassette" and a "Marker Gene" or gene of interest. The RNA expression cassette can be embedded at any permissible sites of

the retroviral construct either as single copy or multiple tandem copies. In addition, although not indicated in the figure, more than one RNA expression cassette may be present in the retroviral construct. Figure 1B shows a similar construct in which the RNA expression cassettes flank a marker gene.

[0027] Figure 2 shows a schematic view of an RNA expression cassette comprising an RNA Polymerase III promoter **100** linked to an RNA coding region **110-130** and a terminator sequence **140**. The RNA coding region comprises a sense region **110**, a loop region **120**, and an antisense region **130**,

[0028] Figure 3 shows a schematic view of an RNA expression cassette having an RNA Polymerase III promoter **100** linked to a first RNA coding region **110** and a first terminator sequence **140** and a second RNA polymerase III promoter **105** linked to a second RNA coding region **115** and a second terminator **145**.

[0029] Figure 4 shows a schematic view of an RNA expression cassette having a first RNA Polymerase III promoter **100** linked to an RNA coding region **110** and a first terminator sequence **145**. The expression cassette has a second RNA polymerase III promoter **105** linked to the RNA coding region **115**, the same sequence as **110** in reverse, and a second terminator **140**.

[0030] Figure 5. Schematic illustration of a lacZ siRNA encoding lentiviral vector. 5'LTR: an HIV based lentiviral vector 5' LTR; F: an HIV Flap element; pol III: a human H1-RNA pol III promoter (-240 to –8); siRNA: a lacZ specific small hairpin RNA coding region and its structure and detailed sequence are illustrated below. UbiC: an internal human ubiquitinC promoter; GFP: a GFP marker gene driven by UbiC promoter. W: a woodchuck RNA regulatory element. 3'LTR: an HIV based self inactivating lentiviral 3' LTR.

[0031] Figure 6. A lacZ specific siRNA encoded by a lentiviral vector can efficiently inhibit the expression of lacZ reporter gene in virus transduced mammalian cells. MEF: mouse embryonic fibroblasts; HEK293: human embryonic kidney cells. Both of the test cell lines harbor lacZ and firefly luciferase reporter genes, and the expression levels of the reporter genes can be measured by chemiluminescent assays. Ctrl: the ratio of lacZ activity versus Luc activity of the uninfected parental cells, which was arbitrarily set to 1. Transduced: the specific inhibition of lacZ expression calculated as the reduction of lacZ to Luc ratio.

[0032] Figure 7. Transgenic animals that express a lacZ specific siRNA molecule encoded by a lentiviral vector can successfully suppress the expression of the ubiquitous lacZ reporter gene in a ROSA26+/- background. ROSA1-6: the lacZ activities in the limb tissues of six E17.5 ROSA26+/- embryos which served as positive controls. The difference in lacZ activity between individual ROSA26+/- embryos may result from variable protein extraction efficiency. TG1-4: the lacZ activities in the limb tissues of four E17.5 transgenic embryos expressing a lentiviral vector-encoded lacZ siRNA molecule in ROSA+/- background. WT1-6: lacZ activity in the limb tissues of six E17.5 C57Bl/6 wildtype embryos, included as the negative control. The background levels of endogenous beta-galactosidase activity are general below 1,000 LU/ug, thus the columns are not visible.

[0033] Figure 8 shows a schematic illustration of a Tet-inducible lacZ siRNA lentiviral vector. A Tet repressor gene (TetR; SEQ ID NO: 5) is the under the control human UbiquitinC promoter and its expression can be monitored by the downstream GFP marker coupled by IRES element (internal ribosomal entry site). The anti-lacZ siRNA cassette is driven by a Tet-inducible pol III promoter derived from human U6-promoter (-328 to +1) containing a single TetR binding site (TetO1) between the PSE and TATA box (SEQ ID NO: 4). In the absence of tetracycline, TetR binds to the promoter and its expression is repressed. Upon the addition of tetracycline, TetR is moved from the promoter and transcription starts.

[0034] Figure 9 shows the results of an experiment that demonstrated that a Tet-inducible siRNA expression cassette can regulate gene expression in response to Doxycycline treatment. lacZ and luciferase double expressing HEK293 cells (293Z+Luc) were transduced with a lentiviral vector carrying a Tet-inducible lacZ-siRNA cassette and a Tet repressor under the control of a UbiquitinC promoter (Figure 8). The transduced cells were treated with 10 ug/ml Doxycycline (Plus Dox) for 48hr or without the Doxycycline treatment as a control (No Dox). LacZ and luciferase activities were measured as described in the previous figures. The relative suppression activity is calculated as the ratio of lacZ versus luciferase and No Dox control was arbitrarily set to 1.

Detailed Description of the Preferred Embodiment

[0035] The inventors have previously identified a method for introducing a transgene of interest into a cell or animal. This technique is described in co-pending U.S.

provisional patent application number 60/322,031 filed on 9/13/2001 and co-pending U.S. provisional patent application number 60/347,782 filed on 1/9/2002, the entire contents of which are incorporated herein by reference.

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Any methods, devices and materials similar or equivalent to those described herein can be used in the practice of this invention.

[0037] By "transgene" is meant any nucleotide sequence, particularly a DNA sequence, that is integrated into one or more chromosomes of a host cell by human intervention, such as by the methods of the present invention. In one embodiment, a transgene is an "RNA coding region." In another embodiment the transgene comprises a "gene of interest." In other embodiments the transgene can be a nucleotide sequence, preferably a DNA sequence, that is used to mark the chromosome where it has integrated. In this situation, the transgene does not have to comprise a gene that encodes a protein that can be expressed.

[0038] A "gene of interest" is a nucleic acid sequence that encodes a protein or other molecule that is desirable for integration in a host cell. In one embodiment, the gene of interest encodes a protein or other molecule the expression of which is desired in the host cell. In this embodiment, the gene of interest is generally operatively linked to other sequences that are useful for obtaining the desired expression of the gene of interest, such as transcriptional regulatory sequences.

[0039] A "functional relationship" and "operably linked" mean, without limitation, that the gene is in the correct location and orientation with respect to the promoter and/or enhancer that expression of the gene will be affected when the promoter and/or enhancer is contacted with the appropriate molecules.

[0040] An "RNA coding region" is a nucleic acid that can serve as a template for the synthesis of an RNA molecule, such as an siRNA. Preferably, the RNA coding region is a DNA sequence.

[0041] A "small interfering RNA" or "siRNA" is a double-stranded RNA molecule that is capable of inhibiting the expression of a gene with which it shares homology. In one embodiment the siRNA may be a "hairpin" or stem-loop RNA molecule, comprising a sense region, a loop region and an antisense region complementary to the

sense region. In other embodiments the siRNA comprises two distinct RNA molecules that are non-covalently associated to form a duplex.

[0042] The term "transgenic" is used herein to describe the property of harboring a transgene. For instance, a "transgenic organism" is any animal, including mammals, fish, birds and amphibians, in which one or more of the cells of the animal contain nucleic acid introduced by way of human intervention, such as by the methods described herein. In a transgenic animal that comprises a transgene that encodes a gene of interest, the transgene typically causes the cell to express or overexpress a recombinant protein. However, according to the methods of the invention, expression of an RNA coding region can be used to down regulate the expression of a particular gene through antisense or RNA interference mechanisms.

[0043] The terms "founder," "founder animal" and "founder line" refer to those animals that are mature products of the embryos or oocytes to which the transgene was added, i.e. those animals that grew from the embryos or oocytes into which DNA was inserted.

[0044] The terms "progeny" and "progeny of the transgenic animal" refer to any and all offspring of every generation subsequent to the originally transformed animal.

[0045] The term "animal" is used in its broadest sense and refers to all animals including mammals, birds, fish, reptiles and amphibians.

[0046] The term "mammal" refers to all members of the class Mammalia and includes any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports or pet animals, such as mouse, rabbit, pig, sheep, goat, cattle and higher primates.

[0047] The term "oocyte" refers to a female gamete cell and includes primary oocytes, secondary oocytes and mature, unfertilized ovum. As used herein, the term "egg" when used in reference to a mammalian egg, means an oocyte surrounded by a zona pellucida. The term "zygote" refers to a fertilized ovum. The term "embryo" broadly refers to an animal in the early stages of development.

[0048] "Perivitelline space" refers to the space located between the zona pellucida and the cell membrane of a mammalian egg or embryonic cell.

[0049] "Target cell" or "host cell" means a cell that is to be transformed using the methods and compositions of the invention.

[0050] "Lentivirus" refers to a genus of retroviruses that are capable of infecting dividing and non-dividing cells. Several examples of lentiviruses include HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2), the etiologic agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, which causes encephalitis (visna) or pneumonia (maedi) in sheep, the caprine arthritis-encephalitis virus, which causes immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia virus, which causes autoimmune hemolytic anemia, and encephalopathy in horses; feline immunodeficiency virus (FIV), which causes immune deficiency in cats; bovine immune deficiency virus (BIV), which causes lymphadenopathy, lymphocytosis, and possibly central nervous system infection in cattle; and simian immunodeficiency virus (SIV), which cause immune deficiency and encephalopathy in sub-human primates.

[0051] A lentiviral genome is generally organized into a 5' long terminal repeat (LTR), the gag gene, the pol gene, the env gene, the accessory genes (nef, vif, vpr, vpu) and a 3' LTR. The viral LTR is divided into three regions called U3, R and U5. The U3 region contains the enhancer and promoter elements. The U5 region contains the polyadenylation signals. The R (repeat) region separates the U3 and U5 regions and transcribed sequences of the R region appear at both the 5' and 3' ends of the viral RNA. See, for example, "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)), O Narayan and Clements J. Gen. Virology 70:1617-1639 (1989), Fields et al. Fundamental Virology Raven Press. (1990), Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. *J Virol.* 72(10):8150-7 (1998), and U.S. Patent No. 6,013,516.

[0052] Lentiviral vectors are known in the art, including several that have been used to transfect hematopoietic stem cells. Such vectors can be found, for example, in the following publications, which are incorporated herein by reference: Evans JT et al. *Hum Gene Ther* 1999;10:1479-1489; Case SS, Price MA, Jordan CT et al. *Proc Natl Acad Sci USA* 1999;96:2988-2993; Uchida N, Sutton RE, Friera AM et al. Proc Natl Acad Sci USA 1998;95:11939-11944; Miyoshi H, Smith KA, Mosier DE et al. *Science* 1999;283:682-686; Sutton RE, Wu HT, Rigg R et al. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J Virol* 1998;72:5781-5788.

[0053] "Virion," "viral particle" and "retroviral particle" are used herein to refer to a single virus comprising an RNA genome, *pol* gene derived proteins, *gag* gene derived proteins and a lipid bilayer displaying an envelope (glyco)protein. The RNA genome is

usually a recombinant RNA genome and thus may contain an RNA sequence that is exogenous to the native viral genome. The RNA genome may also comprise a defective endogenous viral sequence.

[0054] A "pseudotyped" retrovirus is a retroviral particle having an envelope protein that is from a virus other than the virus from which the RNA genome is derived. The envelope protein may be from a different retrovirus or from a non-retroviral virus. A preferred envelope protein is the vesicular stomatitius virus G (VSV G) protein. However, to eliminate the possibility of human infection, viruses can alternatively be pseudotyped with ecotropic envelope protein that limit infection to a specific species, such as mice or birds. For example, in one embodiment, a mutant ecotropic envelope protein is used, such as the ecotropic envelope protein 4.17 (Powell et al. Nature Biotechnology 18(12):1279-1282 (2000)).

[0055] The term "provirus" is used to refer to a duplex DNA sequence present in a eukaryotic chromosome that corresponds to the genome of an RNA retrovirus. The provirus may be transmitted from one cell generation to the next without causing lysis or destruction of the host cell.

[0056] A "self-inactivating 3' LTR" is a 3' long terminal repeat (LTR) that contains a mutation, substitution or deletion that prevents the LTR sequences from driving expression of a downstream gene. A copy of the U3 region from the 3' LTR acts as a template for the generation of both LTR's in the integrated provirus. Thus, when the 3' LTR with an inactivating deletion or mutation integrates as the 5' LTR of the provirus, no transcription from the 5' LTR is possible. This eliminates competition between the viral enhancer/promoter and any internal enhancer/promoter. Self-inactivating 3' LTRs are described, for example, in Zufferey et al. *J. Virol.* 72:9873-9880 (1998), Miyoshi et al. *J. Virol.* 72:8150-8157 and Iwakuma et al. *Virology* 261:120-132 (1999).

[0057] The term "RNA interference or silencing" is broadly defined and includes all posttranscriptional and transcriptional mechanisms of RNA mediated inhibition of gene expression, such as those described in (P.D. Zamore *Science* 296, 1265 (2002)).

[0058] In one aspect of the invention, a recombinant retrovirus is used to deliver a transgene comprising an RNA coding region of interest to a target cell. Preferably the target cell is a mammalian cell. The cell may be a primary cell, or may be a cultured cell, for example an without limitation an HEK, CHO, COS, MEF, 293 cell. In one embodiment

the target cell is an oocyte or an embryonic cell, more preferably a one-cell embryo. The RNA coding region and any associated genetic elements are thus integrated into the genome of the target cell as a provirus. When the target cell is an embryo, the cell may then be allowed to develop into a transgenic animal by methods well known in the art.

[0059] The recombinant retrovirus used to deliver the RNA coding region is preferably a modified lentivirus, and thus is able to infect both dividing and non-dividing cells. The recombinant retrovirus preferably comprises a modified lentiviral genome that includes the transgene. Further, the modified lentiviral genome preferably lacks endogenous genes for proteins required for viral replication, thus preventing undesired replication, such as replication in a resulting transgenic animal. The required proteins are preferably provided in *trans* in the packaging cell line during production of the recombinant retrovirus, as described below.

[0060] In another embodiment, the recombinant retrovirus used to deliver the RNA coding region is a modified Moloney virus, for example a Moloney Murine Leukemia Virus. In a further embodiment, the virus is a Murine Stem Cell Virus (Hawley, R. G., et al. (1996) Proc. Natl. Acad. Sci. USA 93:10297–10302; Keller, G., et al. (1998) Blood 92:877–887; Hawley, R. G., et al. (1994) Gene Ther. 1:136–138). The recombinant retrovirus also can be a hybrid virus such as that described in Choi, JK; Hoanga, N; Vilardi, AM; Conrad, P; Emerson, SG; Gewirtz, AM. (2001) Hybrid HIV/MSCV LTR Enhances Transgene Expression of Lentiviral Vectors in Human CD34+ Hematopoietic Cells. *Stem Cells* **19**, No. 3, 236-246.

[0061] In the preferred embodiment the transgene is incorporated into a viral construct that comprises an intact retroviral 5' LTR and a self-inactivating 3' LTR. The viral construct is preferably introduced into a packaging cell line that packages viral genomic RNA based on the viral construct into viral particles with the desired host specificity. Viral particles are collected and used to infect the host cell. Each of these aspects is described in detail below.

The Viral Construct

[0062] The viral construct is a nucleotide sequence that comprises sequences necessary for the production of recombinant retrovirus in a packaging cell. In one

embodiment the viral construct additionally comprises genetic elements that allow for the desired expression of an RNA molecule or gene of interest in the host.

[0063] Generation of the viral construct can be accomplished using any suitable genetic engineering techniques well known in the art, including, without limitation, the standard techniques of PCR, oligonucleotide synthesis, restriction endonuclease digestion, ligation, transformation, plasmid purification, and DNA sequencing, for example as described in Sambrook et al. (Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, N.Y. (1989)), Coffin et al. (Retroviruses. Cold Spring Harbor Laboratory Press, N.Y. (1997)) and "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)).

[0064] The viral construct may incorporate sequences from the genome of any known organism. The sequences may be incorporated in their native form or may be modified in any way. For example, the sequences may comprise insertions, deletions or substitutions. In the preferred embodiment the viral construct comprises sequences from a lentivirus genome, such as the HIV genome or the SIV genome.

[0065] The viral construct preferably comprises sequences from the 5' and 3' LTRs of a lentivirus. More preferably the viral construct comprises the R and U5 sequences from the 5' LTR of a lentivirus and an inactivated or self-inactivating 3' LTR from a lentivirus. The LTR sequences may be LTR sequences from any lentivirus from any species. For example, they may be LTR sequences from HIV, SIV, FIV or BIV. Preferably the LTR sequences are HIV LTR sequences. The virus also can incorporate sequences from MMV or MSCV.

[0066] The viral construct preferably comprises an inactivated or self-inactivating 3' LTR. The 3' LTR may be made self-inactivating by any method known in the art. In the preferred embodiment the U3 element of the 3' LTR contains a deletion of its enhancer sequence, preferably the TATA box, Sp1 and NF-kappa B sites. As a result of the self-inactivating 3' LTR, the provirus that is integrated into the host cell genome will comprise an inactivated 5' LTR.

[0067] Optionally, the U3 sequence from the lentiviral 5' LTR may be replaced with a promoter sequence in the viral construct. This may increase the titer of virus recovered from the packaging cell line. An enhancer sequence may also be included. Any enhancer/promoter combination that increases expression of the viral RNA genome in the

packaging cell line may be used. In the preferred embodiment the CMV enhancer/promoter sequence is used (U.S. Patent No. 5,168,062; Karasuyama et al J. Exp. Med. 169:13 (1989).

[0068] The viral construct also comprises a transgene. The transgene, may be any nucleotide sequence, including sequences that serve as markers for the provirus. Preferably the transgene comprises one or more RNA coding regions and/or one or more genes of interest. Schematic diagrams of exemplary retroviral constructs are shown in Figures 1A and 1B.

[0069] In the preferred embodiment the transgene comprises at least one RNA coding region. Preferably the RNA coding region is a DNA sequence that can serve as a template for the expression of a desired RNA molecule in the host cell. In one embodiment, the viral construct comprises two or more RNA coding regions.

[0070] The viral construct also preferably comprises at least one RNA Polymerase III promoter. The RNA Polymerase III promoter is operably linked to the RNA coding region and can also be linked to a termination sequence. In addition, more than one RNA Polymerase III promoter may be incorporated.

[0071] RNA Polymerase III promoters are well known to one of skill in the art. A suitable range of RNA Polymerase III promoters can be found, for example, in Paule and White. *Nucleic Acids Research.*, Vol 28, pp 1283-1298 (2000), which is hereby incorporated by reference in its entirety. The definition of RNA Polymerase III promoters also include any synthetic or engineered DNA fragment that can direct RNA Polymerase III to transcribe a downstream RNA coding sequence. Further, the RNA Polymerase III (Pol III) promoter or promoters used as part of the viral vector can be inducible. Any suitable inducible Pol III promoter can be used with the methods of the invention. Particularly suited Pol III promoters include the tetracycline responsive promoters provided in Ohkawa and Taira *Human Gene Therapy*, Vol. 11, pp 577-585 (2000) and in Meissner *et al. Nucleic Acids Research*, Vol. 29, pp 1672-1682 (2001), which are incorporated herein by reference.

[0072] In one embodiment the transgene comprises a gene of interest that encodes a protein that is desirably expressed in one or more cells of a transgenic animal, for example, a reporter or marker protein. Preferably the gene of interest is located between the 5' LTR and 3' LTR sequences. Further, the gene of interest is preferably in a functional relationship with other genetic elements, for example transcription regulatory sequences such as promoters and/or enhancers, to regulate expression of the gene of interest in a

particular manner once the transgene is incorporated into the host genome. In certain embodiments, the useful transcriptional regulatory sequences are those that are highly regulated with respect to activity, both temporally and spatially.

[0073] Preferably the gene of interest is in a functional relationship with internal Polymerase II promoter/enhancer regulatory sequences. An "internal" promoter/enhancer is one that is located between the 5' LTR and the 3' LTR sequences in the viral construct and is operably linked to the gene that is desirably expressed.

[0074] The Polymerase II promoter/enhancer may be any promoter, enhancer or promoter/enhancer combination known to increase expression of a gene with which it is in a functional relationship.

[0075] The internal promoter/enhancer is preferably selected based on the desired expression pattern of the gene of interest and the specific properties of known promoters/enhancers. Thus, the internal promoter may be a constitutive promoter. Non-limiting examples of constitutive promoters that may be used include the promoter for ubiquitin, CMV (U.S. Patent No. 5,168,062; Karasuyama et al J. Exp. Med. 169:13 (1989), ß-actin (Gunning et al. Proc. Natl. Acad. Sci. USA 84:4831-4835 (1987) and pgk (see, for example, U.S. Patent Nos. 4,615,974 and 5,104,795; Adra et al. Gene 60:65-74 (1987), Singer-Sam et al. Gene 32:409-417 (1984) and Dobson et al. Nucleic Acids Res. 10:2635-2637 (1982)). Alternatively, the promoter may be a tissue specific promoter. Several non-limiting examples of tissue specific promoters that may be used include lck (see, for example, Garvin et al. Mol. Cell Biol. 8:3058-3064 (1988) and Takadera et al. Mol. Cell Biol. 9:2173-2180 (1989)), myogenin (Yee et al. Genes and Development 7:1277-1289 (1993), and thyl (Gundersen et al. Gene 113:207-214 (1992). In addition, promoters may be selected to allow for inducible expression of the transgene. A number of systems for inducible expression using such a promoter are known in the art, including the tetracycline responsive system and the *lac* operator-repressor system. It is also contemplated that a combination of promoters may be used to obtain the desired expression of the gene of interest. The skilled artisan will be able to select a promoter based on the desired expression pattern of the gene in the resulting transgenic animal.

[0076] An internal enhancer may also be present in the viral construct to increase expression of the gene of interest. For example the CMV enhancer (Karasuyama et al J. Exp. Med. 169:13 (1989) may be used in combination with the chicken ß-actin

promoter (see, e.g., JP 1990005890-A1). Again, one of skill in the art will be able to select the appropriate enhancer based on the desired expression pattern.

[0077] The gene of interest is not limited in any way and includes any gene that the skilled practitioner desires to have integrated and/or expressed in a transgenic animal. For example, the gene of interest may be one that encodes a protein that serves as a marker to identify cells comprising the provirus. In other embodiments the gene of interest encodes a protein that modifies a physical characteristic of the transgenic animal, such as a protein that modifies size, growth, or tissue composition. In another example the gene of interest may encode a protein of commercial value that may be harvested from the transgenic animal.

[0078] In addition, more than one gene of interest may be placed in functional relationship with the internal promoter. For example a gene encoding a marker protein may be placed after the primary gene of interest to allow for identification of cells that are expressing the desired protein. In one embodiment a fluorescent marker protein, preferably green fluorescent protein (GFP), is incorporated into the construct along with the gene of interest. If a second reporter gene is included, an internal ribosomal entry site (IRES) sequence is also preferably included (U.S. Patent No. 4,937,190). The IRES sequence may facilitate the expression of the reporter gene.

[0079] The viral construct may also contain additional genetic elements. The types of elements that may be included in the construct are not limited in any way and will be chosen by the skilled practitioner to achieve a particular result. For example, a signal that facilitates nuclear entry of the viral genome in the target cell may be included. An example of such a signal is the HIV-1 flap signal.

[0080] Further, elements may be included that facilitate the characterization of the provirus integration site in the genome of the animal. For example, a tRNA amber suppressor sequence may be included in the construct.

[0081] In addition, the construct may contain one or more genetic elements designed to enhance expression of the gene of interest. For example, a woodchuck hepatitis virus responsive element (WRE) may be placed into the construct (Zufferey et al. *J. Virol.* 74:3668-3681 (1999); Deglon et al. *Hum. Gene Ther.* 11:179-190 (2000)).

[0082] A chicken β-globin insulator (Chung et al. *Proc. Natl. Acad. Sci. USA* 94:575-580 (1997)) may also be included in the viral construct. This element has been

shown to reduce the chance of silencing the integrated provirus in the transgenic animal due to methylation and heterochromatinization effects. In addition, the insulator may shield the internal enhancer, promoter and exogenous gene from positive or negative positional effects from surrounding DNA at the integration site on the chromosome.

[0083] Any additional genetic elements are preferably inserted 3' of the gene of interest.

[0084] In a specific embodiment, the viral vector comprises: a cytomegalovirus (CMV) enhancer/promoter sequence; the R and U5 sequences from the HIV 5' LTR; the HIV-1 flap signal; an internal enhancer; an internal promoter; a gene of interest; the woodchuck hepatitis virus responsive element; a tRNA amber suppressor sequence; a U3 element with a deletion of its enhancer sequence; the chicken β-globin insulator; and the R and U5 sequences of the 3' HIV LTR.

[0085] The viral construct is preferably cloned into a plasmid that may be transfected into a packaging cell line. The preferred plasmid preferably comprises sequences useful for replication of the plasmid in bacteria.

Production of Virus

[0086] Any method known in the art may be used to produce infectious retroviral particles whose genome comprises an RNA copy of the viral construct described above.

[0087] Preferably, the viral construct is introduced into a packaging cell line. The packaging cell line provides the viral proteins that are required in *trans* for the packaging of the viral genomic RNA into viral particles. The packaging cell line may be any cell line that is capable of expressing retroviral proteins. Preferred packaging cell lines include 293 (ATCC CCL X), HeLa (ATCC CCL 2), D17 (ATCC CCL 183), MDCK (ATCC CCL 34), BHK (ATCC CCL-10) and Cf2Th (ATCC CRL 1430). The most preferable cell line is the 293 cell line.

[0088] The packaging cell line may stably express the necessary viral proteins. Such a packaging cell line is described, for example, in U.S. Patent No. 6,218,181. Alternatively a packaging cell line may be transiently transfected with plasmids comprising nucleic acid that encodes the necessary viral proteins.

[0089] In one embodiment a packaging cell line that stably expresses the viral proteins required for packaging the RNA genome is transfected with a plasmid comprising the viral construct described above.

[0090] In another embodiment a packaging cell line that does not stably express the necessary viral proteins is co-transfected with two or more plasmids essentially as described in Yee et al. (*Methods Cell. Biol.* 43A, 99-112 (1994)). One of the plasmids comprises the viral construct comprising the transgene. The other plasmid(s) comprises nucleic acid encoding the proteins necessary to allow the cells to produce functional virus that is able to infect the desired host cell.

[0091] The packaging cell line may not express envelope gene products. In this case the packaging cell line will package the viral genome into particles that lack an envelope protein. As the envelope protein is responsible, in part, for the host range of the viral particles, the viruses are preferably pseudotyped. Thus the packaging cell line is preferably transfected with a plasmid comprising sequences encoding a membrane-associated protein that will permit entry of the virus into a host cell. One of skill in the art will be able to choose the appropriate pseudotype for the host cell that is to be used. For example, in one embodiment the viruses are pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSVg). In addition to conferring a specific host range this pseudotype may permit the virus to be concentrated to a very high titer. Viruses can alternatively be pseudotyped with ecotropic envelope proteins that limit infection to a specific species, such as mice or birds. For example, in another embodiment, a mutant ecotropic envelope protein is used, such as the ecotropic envelope protein 4.17 (Powell et al. Nature Biotechnology 18(12):1279-1282 (2000)).

[0092] In the preferred embodiment a packaging cell line that does not stably express viral proteins is transfected with the viral construct, a second vector comprising the HIV-1 packaging vector with the *env*, *nef*, 5'LTR, 3'LTR and vpu sequences deleted, and a third vector encoding an envelope glycoprotein. Preferably the third vector encodes the VSVg envelope glycoprotein.

[0093] In another embodiment of invention, RNA interference activity of the packaging cells is suppressed to improve the production of recombinant virus. This includes, without limitation, the use of cotransfection or stable transfection of constructs expressing siRNA molecules to inhibit Dicer, an RNase III family member of ribonuclease

which is essential for RNA interference (Hammond et al. Nat. Rev. Genet. 2:110-119 (2001)).

[0094] The recombinant virus is then preferably purified from the packaging cells, titered and diluted to the desired concentration.

Transgenic Animals

[0095] In order to make transgenic animals, an oocyte or one or more embryonic cells are infected with the recombinant virus produced as described above. One of skill in the art will recognize that the method of infection and the treatment of the cell following infection will depend upon the type of animal from which the cell is obtained. For example, mammalian cells are preferably implanted in a pseudopregnant female following infection while for the generation of transgenic birds or fish, the virus is preferably delivered to a laid egg and thus implantation is not required.

[0096] While early methods of making transgenic animals required the cells to be rapidly dividing, there is no such requirement in the methods of the present invention. Thus the cell may be contacted at any point in development. In the preferred embodiment, a zygote is contacted with the recombinant virus.

[0097] The cells to be infected with the virus may be obtained by any method known in the art and appropriate for the specific species in which it is desired to make a transgenic animal. For example, the recovery of fertilized mouse oocytes is described in Hogan et al. (Manipulating the Mouse Embryo: A Laboratory Manual. 2^{nd} ed. Cold Spring Harbor Laboratory Press, NY (1994)). A method for obtaining fertilized rat oocytes is described in Armstrong et al. (Biol. Reprod. 39,511-518 (1998)).

[0098] It is not necessary that the cells be contacted after fertilization. In one embodiment, the virus is delivered to unfertilized ova. Development may then be initialized, for example by *in vitro* fertilization.

Delivery of the Virus

[0099] The virus may be delivered to the cell in any way that allows the virus to infect the cell. Preferably the virus is allowed to contact the cell membrane. Two preferred methods of delivering the virus to mammalian cells, injection and direct contact, are described below.

Injection

[0100] In a first embodiment the virus is injected into the perivitelline space between the zona pellucida and the cell membrane of a single-cell zygote. Preferably less than 50 picoliters of viral suspension is injected, more preferably less than 25 picoliters and even more preferably about 10 picoliters.

[0101] The virus is preferably present in a viral suspension and may be injected by any method known in the art. The viral suspension is preferably injected through a hydraulic injector. More preferably a glass micropipette is used to inject the virus. In one embodiment a micropipette is prepared by pulling borosilicate glass capillary on a pipette puller. The tip is preferably opened and beveled to approximately 10 μm. The lentiviral suspension may be loaded into the micropipette from the tip using gentle negative pressure.

[0102] In one embodiment the cell is stabilized with a holding pipette mounted on a micromanipulator, such as by gentle negative pressure against a fire-polished pipette, and a second micromanipulator is used to direct the tip of a micropipette into the space between the zona pellucida and the cell membrane, where the virus is injected.

Direct contact

[0103] In another embodiment the zona pellucida is removed from the cell to produce a denuded embryo and the cell membrane is contacted with the virus. The zona pellucida may be removed by any method known in the art. Preferably it is removed by enzymatic treatment. For example, treatment with pronase may be used to remove the zona pellucida while the cell membrane is kept intact. Alternatively, the cell may be placed in media at pH at which the zona pellucida dissolves while the cell membrane remains intact. For example the cell may be incubated in an acidic Tyrode's solution at room temperature for several minutes. Once the zona pellucida is removed, any method that allows for the virus to contact the cell membrane may be used. Preferably, the cell is incubated in a solution containing the virus. Even more preferably, the solution is media that facilitates survival of the cell.

[0104] In this embodiment, the cells are preferably contacted with the virus in culture plates. The virus may be suspended in media and added to the wells of a multi-well culture plate. The cells may then be plated in the individual wells. The media containing the virus may be added prior to the plating of the cells or after the cells have been plated.

Preferably individual cells are incubated in approximately 10 μl of media. However, any amount of media may be used as long as an appropriate concentration of virus in the media is maintained such that infection of the host cell occurs.

[0105] The cells are preferably incubated with the virus for a sufficient amount of time to allow the virus to infect the cells. Preferably the cells are incubated with virus for at least 1 hour, more preferably at least 5 hours and even more preferably at least 10 hours.

[0106] Both the injection and direct contact embodiments may advantageously be scaled up to allow high throughput transgenesis. Because of the relative simplicity of the injection technique, it is possible to inject many embryos rapidly. For example, it is possible to inject more than 200 fertilized oocytes in less than one hour. With regard to the direct contact embodiment, any number of embryos may be incubated in the viral suspension simultaneously. This may be accomplished, for example, by plating the desired number of single-cell zygotes in multi-well tissue culture plates containing the virus suspended in media appropriate for the survival and growth of the cells.

[0107] In both embodiments, any concentration of virus that is sufficient to infect the cell may be used. Preferably the concentration is at least 1 pfu/μl, more preferably at least 10 pfu/μl, even more preferably at least 400 pfu/μl and even more preferably at least 1×10^4 pfu/μl.

[0108] Following infection with the virus, the cells are preferably implanted in an animal. More preferably cells infected with the virus are implanted in pseudo-pregnant animals of the same species from which the infected cells were obtained. Methods of creating pseudo-pregnancy in animals and implanting embryos are well known in the art and are described, for example, in Hogan et al. (Manipulating the Mouse Embryo: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, NY (1994)).

[0109] In the preferred embodiment early stage embryos (approximately 0 - 2.5 days p.c.) still with an intact zona pellucida are transferred to the oviduct of timed pseudopregnant female (preferably 0.5 days p.c.), while embryos that have reached the blastocyst stage are transferred to the uterus of timed pseudopregnant females (preferably 2.5 days p.c.). Denuded embryos are preferably cultured in vitro until they reach the morula or blastocyst stage (48 to 72 hours in culture), and are then implanted into appropriately timed pseudopregnant females.

[0110] The embryos and resulting animals may be analyzed, for example for integration of the transgene, the number of copies of the transgene that integrated, the location of the integration, the ability to transmit the transgene to progeny and expression of the transgene. Such analysis may be carried out at any time and may be carried out by any methods known in the art. Standard techniques are described, for example, in Hogan et al. (supra).

[0111] The methods of infecting cells disclosed above do not depend upon species-specific characteristics of the cells. As a result, they are readily extended to all mammalian species.

[0112] Initial experiments with mice indicate that of those animals that develop to full term, 80-90% carried at least one copy of the transgene and that, of these, approximately 85% express the gene of interest. Of the transgenic animals about 25% carry only 1 or 2 copies of the transgene. The highest number of proviral insertions observed was about 30. Of the animals that carried only 1 or 2 copies of the transgene, about 80% expressed the gene of interest.

[0113] As discussed above, the modified retrovirus can be pseudotyped to confer upon it a broad host range. One of skill in the art would also be aware of appropriate internal promoters to achieve the desired expression of a gene of interest in a particular animal species. Thus, one of skill in the art will be able to modify the method of infecting cells to create transgenic animals of any species.

[0114] In one embodiment, transgenic birds are created by delivering a modified retrovirus, as described above, to the primordial germ cells of early stage avian embryos. Freshly laid eggs are obtained and placed in a temperature controlled, humidified incubator. Preferably, the embryonic blastodisc in the egg is gradually rotated to lie on top of the yolk. This may be accomplished by any method known in the art, such as by gently rocking the egg regularly, preferably every 15 minutes. Approximately 36 hours later, the modified retrovirus is delivered into the space between the embryonic disk and the perivitelline membrane. Preferably about 50 nL of viral solution is delivered, more preferably about 100 nL of viral solution is delivered, and even more preferably about 200 nL of viral solution is delivered. The viral solution may be delivered by any method known in the art for delivering compositions to the inside of an egg. In the preferred embodiment a window is opened in the shell, the viral solution is injected through the window and the shell window

is closed. The eggs are preferably incubated until hatching. The eggs will hatch after approximately 20 days, depending upon the particular avian species from which they are obtained. Hatched chicks are preferably raised to sexual maturity and mated. The transgenic offspring of the founder animals may be identified by any method known in the art, such as Southern blot, PCR and expression analysis.

[0115] In another embodiment, transgenic fish are created by delivering the modified retrovirus, described above, to single-cell fish embryos. Fertilized fish eggs are collected by any method known in the art. The modified retrovirus is then preferably delivered to the space between the chorion and the cell membrane. This may be accomplished, for example, by loading the modified retrovirus in solution into a glass pipette. The pipette may then be used to pierce the chorion membrane and deliver the viral suspension. Preferably about 50 nL of viral solution is delivered, more preferably about 100 nL of viral solution is delivered, and even more preferably about 200 nL of viral solution is delivered. Injected embryos are preferably returned to a temperature-controlled water tank and allowed to mature. At sexual maturity the founder fish are preferably mated and their progeny analyzed for the presence of the transgene by any method known in the art.

[0116] As mentioned above, the methods of the present invention will also prove useful in techniques for identifying genes that are involved in specific biological processes, such as gene trap assays and large-scale mutagenesis screens. Such methods are described in the copending provisional patent applications 60/322,031 filed on 9/13/2001 and copending U.S. provisional patent application 60/347,782 filed on 1/9/2002.

Down-regulating Gene Expression in a Target Cell

[0117] The methods described herein allow the expression of RNA molecules in cells, and are particularly suited to the expression of small RNA molecules, which can not be readily expressed from a Pol II promoter. According to a preferred embodiment of the invention, an RNA molecule is expressed within a cell in order to down-regulate the expression of a target gene. The ability to down-regulate a target gene has many therapeutic and research applications, including identifying the biological functions of particular genes. Using the techniques and compositions of the invention, it will be

possible to knock-down (or down-regulate) the expression of a large number of genes, both in cell culture and in mammalian organisms.

[0118] In preferred embodiments of the invention, an RNA expression cassette comprises a Pol III promoter and an RNA coding region. The RNA coding region preferably encodes an RNA molecule that is capable of down-regulating the expression of a particular gene or genes. The RNA molecule encoded can, for example, be complementary to the sequence of an RNA molecule encoding a gene to be down-regulated. In such an embodiment, the RNA molecule preferably acts through an antisense mechanism.

[0119] A more preferred embodiment involves the expression of a double-stranded RNA complex, or an RNA molecule having a stem-loop or a so-called "hairpin" structure. As used herein, the term "RNA duplex" refers to the double stranded regions of both the RNA complex and the double-stranded region of the hairpin or stem-lop structure.

[0120] Double stranded RNA has been shown to inhibit gene expression of genes having a complementary sequence through a process termed RNA interference or suppression (see, for example, Hammond et al. Nat. Rev. Genet. 2:110-119 (2001)).

[0121] According to the invention, an RNA duplex or siRNA corresponding to a region of a gene to be down-regulated is expressed in the cell. The RNA duplex is substantially identical (typically at least about 80% identical, more preferably at least about 90% identical) in sequence to the sequence of the gene targeted for down regulation. siRNA duplexes are described, for example, in Bummelkamp et al. Science 296:550-553 (2202), Caplen et al. Proc. Natl. Acad. Sci. USA 98:9742-9747 (2001) and Paddison et al. Genes & Devel. 16:948-958 (2002).

[0122] The RNA duplex is generally at least about 15 nucleotides in length and is preferably about 15 to about 30 nucleotides in length. However, a significantly longer RNA duplex can be used effectively in some organisms. In a more preferred embodiment, the RNA duplex is between about 19 and 22 nucleotides in length. The RNA duplex is preferably identical to the target nucleotide sequence over this region.

[0123] When the gene to be down regulated is in a family of highly conserved genes, the sequence of the duplex region can be chosen with the aid of sequence comparison to target only the desired gene. On the other hand, if there is sufficient identity among a family of homologous genes within an organism, a duplex region can be designed that would down regulate a plurality of genes simultaneously.

[0124] The duplex RNA can be expressed in a cell from a single retroviral construct. In the preferred embodiment, a single RNA coding region in the construct is a serves as a template for the expression of a self-complementary hairpin RNA, comprising a sense region, a loop region and an antisense region. This embodiment is illustrated in Figure 2, which shows a schematic view of an RNA expression cassette having an RNA Pol III promoter **100** operatively linked to an RNA coding region, having a sense region **110**, a loop region **120**, an antisense region **130** and a terminator region **140**. The sense **110** and antisense **130** regions are each preferably about 15 to about 30 nucleotides in length. The loop region **120** preferably is about 2 to about 15 nucleotides in length, more preferably from about 4 to about 9 nucleotides in length. Following expression the sense and antisense regions form a duplex.

[0125] In another embodiment, the retroviral construct comprises two RNA coding regions. The first coding region is a template for the expression of a first RNA and the second coding region is a template for the expression of a second RNA. Following expression, the first and second RNA's form a duplex. The retroviral construct preferably also comprises a first Pol III promoter operably linked to the first RNA coding region and a second Pol III promoter operably linked to the second RNA coding region. This embodiment is illustrated in Figure 3, which shows a schematic view of an RNA expression cassette having an RNA Polymerase III promoter **100** linked to a first RNA coding region **110** and a first terminator sequence **140** and a second RNA polymerase III promoter **105** linked to a second RNA coding region **115** and a second terminator **145**.

[0126] In yet another embodiment of the invention, the retroviral construct comprises a first RNA Pol III promoter operably linked to a first RNA coding region, and a second RNA Pol III promoter operably linked to the same first RNA coding region in the opposite direction, such that expression of the RNA coding region from the first RNA Pol III promoter results in a synthesis of a first RNA molecule as the sense strand and expression of the RNA coding region from the second RNA Pol III promoter results in synthesis of a second RNA molecule as an antisense strand that is substantially complementary to the first RNA molecule. In one such embodiment, both RNA Polymerase III promoters are separated from the RNA coding region by termination sequences, preferably termination sequences having five consecutive T residues. Figure 4 shows a schematic view of an RNA expression cassette having a first RNA Polymerase III

promoter **100** linked to an RNA coding region **110** and a first terminator sequence **145**. The expression cassette has a second RNA polymerase III promoter **105** linked to the RNA coding region **115**, the same sequence as **110** in reverse, and a second terminator **140**.

[0127] In further embodiments an RNA duplex is expressed using two or more retroviral constructs. In one embodiment, a first retroviral construct is used that directs the expression of a first RNA and a second retroviral construct is used that directs expression of a second RNA that is complementary to the first. Following expression the first and second RNAs form a duplex region. It is preferred, however, that the entire duplex region is introduced using retroviral particles derived from a single retroviral construct. As discussed above, several strategies for expressing a duplex RNA from a single viral construct are shown in Figures 2-4.

[0128] The RNA duplexes may be flanked by single stranded regions on one or both sides of the duplex. For example, in the case of the hairpin, the single stranded loop region would connect the duplex region at one end.

[0129] The RNA coding region is generally operatively linked to a terminator sequence. The pol III terminators preferably comprise of stretches of 4 or more thymidine ("T") residues. In a preferred embodiment, a cluster of 5 consecutive Ts is linked immediately downstream of the RNA coding region to serve as the terminator. In such a construct pol III transcription is terminated at the second or third T of the DNA template, and thus only 2 to 3 uridine ("U") residues are added to the 3' end of the coding sequence.

[0130] The sequence of the RNA coding region, and thus the sequence of the RNA duplex, preferably is chosen to be complementary to the sequence of a gene whose expression is to be downregulated in a cell or organism. The degree of down regulation achieved with a given RNA duplex sequence for a given target gene will vary by sequence. One of skill in the art will be able to readily identify an effective sequence. For example, in order to maximize the amount of suppression in a transgenic animal, a number of sequences can be tested for their efficacy in cell culture prior to generating a transgenic animal.

[0131] The methods of the present invention will find great commercial application, for example in biotechnology, medicine and agriculture. For example, in agriculture the described methods may be used to confer disease resistance by expressing in a cell or organism an siRNA that specifically down-regulates the expression of a gene

associated with a pathogen or disease state. In biotechnology, the ability to rapidly develop large numbers of transgenic animals with desired modulation of specific genes will allow for the analysis of gene function and the evaluation of compounds that potentially modulate gene expression, protein function, and are useful in treating a disease or disorder. In particular, by observing the effect of down-regulating specific genes in transgenic animals, the biological function of those genes may be determined. In medicine the methods of the invention may be used to treat patients suffering from particular diseases or disorders, such as HIV, or to confer immunity or resistance to particular pathogens. For example, specific cells may be infected in vivo or ex vivo with recombinant retrovirus encoding an siRNA that down-regulates the activity of a gene whose activity is associated with a particular disease or disorder.

[0132] The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

[0133] All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

Examples

Example 1

[0134] An lentiviral construct was constructed by insertion of an siRNA expression cassette into the PacI site of HC-FUGW vector (Figure 5; SEQ ID NO: 2). The siRNA was designed to down-regulate expression of the lacZ gene. The HC-FUGW vector comprised a GFP marker gene operably linked to the human Ubiquitin promoter. The GFP marker was useful for tracking transduction events. The vector also comprised an HIV DNA Flap element to improve the virus titers, and the WRE for high level expression of viral genes. The siRNA expression cassette was composed of a pol III promoter and a small hairpin RNA coding region followed by a pol III terminator site. The pol III promoter (SEQ ID NO:3) was derived from the –240 to -9 region of human H1-RNA promoter and

was cloned as an Eco R1 fragment by PCR amplification from HEK293 genomic DNA. The pol III promoter was connected to the downstream RNA coding region by a 7 base pair linker sequence to ensure that the transcription was precisely initiated at the first nucleotide of the RNA coding sequence. The small hairpin RNA coding region comprised a 19 nt sequence corresponding to the 1900-1918 region of the sense strand of the bacterial beta-galactosidase (lacZ) gene coding sequence and the 19 nt perfect reverse complementary sequence separated by a 9 nt loop region. The terminator was comprised of 5 consecutive thymidine residues linked immediately downstream of the RNA coding sequence. The sequence of the hairpin siRNA is shown in SEQ ID NO: 1.

Example 2

[0135] Transduction of cultured mammalian cells with retrovirus derived from the retroviral construct described in Example 1 was achieved (Figure 6). The retroviral vector encoding a small hairpin RNA molecule described in Example 1, was used to transfect cultured mammalian cells that express lacZ. A profound decrease in the expression of the lacZ was observed.

[0136] The lacZ siRNA virus was produced by cotransfection of the retroviral vector, a helper virus plasmid and VSVg expression plasmid in HEK293 cells. The virus particles were harvested from the cell culture supernatants and concentrated by ultracentrifugation. The concentrated virus preparations were used to infect either mouse embryonic fibroblasts (MEF) or HEK293 cells which harbor both lacZ and firefly luciferase (Luc) reporter genes. Infection was monitored by the GFP signal which is expressed from the marker gene cassette of the viral vector. Under the conditions of this experiment, >98% of the test cells were GPF+ and thus were successfully transduced. The expression levels of lacZ and Luc reporter genes were measured by chemiluminescent assays using commercially available kits (lacZ assay kit from Roche and Luc from Promega). The lacZ siRNA virus only inhibited the expression of lacZ but not Luc. The specific inhibition was determined by the ratio of lacZ activity to Luc activity. The lacZ/Luc ratio of the uninfected parental cells was arbitrarily set to 1 and the values of the infected cells were calculated accordingly. As shown in Figure 6, transfection with the virus resulted in dramatic reduction in the amount of expression of the lacZ gene in both MEK and HEK293 cells.

[0137] A tet-iducible lacZ siRNA lentiviral vector was also prepared as illustrated in Figure 8. A Tet repressor gene (TetR; SEQ ID NO: 5) was placed the under the control of the human UbiquitinC promoter so that its expression could be monitored by the downstream GFP marker. The anti-lacZ siRNA cassette was driven by a Tet-inducible pol III promoter derived from human U6-promoter (-328 to +1) containing a single TetR binding site (TetO1) between the PSE and TATA box (SEQ ID NO: 4). The TetR coding sequence was PCR amplified from genomic DNA from the TOP10 strain of E. coli adn cloned into a modified version of FUIGW as a Bgl2-EcoR1 fragment. In the absence of tetracycline, TetR binds to the promoter and its expression is repressed. Upon the addition of tetracycline, TetR is moved from the promoter and transcription starts.

[0138] The Tet-inducible siRNA expression cassette was able to regulate gene expression in response to Doxycycline treatment. Virus was prepared from the retroviral construct carrying the Tet-inducible lacZ-siRNA cassette and a Tet repressor under the control of a UbiquitinC promoter and used to transduce HEK293 cells expressing both lacZ and luciferase (293Z+Luc). The transduced cells were treated with 10 ug/ml Doxycycline (Plus Dox) for 48hr or without the Doxycycline treatment as a control (No Dox). LacZ and luciferase activities were measured as described in the previous figures. The relative suppression activity is calculated as the ratio of lacZ versus luciferase and No Dox control was arbitrarily set to 1. As can be seen in Figure 9, in the presence of doxycycline suppression of lacZ activity was significantly enhanced.

Example 3

[0139] This example demonstrates the generation of transgenic animals that express an siRNA molecule encoded by a lentiviral vector. The expression of the lacZ specific siRNA described in Example 1 resulted in extensive suppression of lacZ activity in ROSA26+/- mice.

[0140] ROSA26+/- mice carry one copy of a ubiquitously expressed lacZ reporter gene. The lacZ siRNA virus preparations described in Example 2 were used for perivitelline injection of ROSA26+/- single cell embryos obtained from hormone primed C57Bl/6 female donors x ROSA26+/+ stud males. The injected single cell embryos were subsequently transferred into the oviduct of timed pseudopregnant female recipients. Embryonic day 15.5 to 17.5 (E15.5-17.5) fetuses were recovered from the surrogate

mothers. Successful transgenesis was scored by positive GFP signal observed with the fetuses under fluorescent microscope. Protein extracts prepared from the limb tissues of the fetuses were used for the LacZ chemiluminescent assay according to the manufacturer's instruction (Roche), and protein concentrations of the tissue extracts were determined by the Bradford assay (BioRad). The lacZ expression levels were expressed as light units (LU) per ug of proteins (LU/ug). The E15.5-17.5 fetuses from the timed mating of C57Bl/6 females x ROSA26+/+ males and C57Bl/6 females x C57Bl/6 males were served as the positive and negative controls respectively. The results are shown in Figure 7. Animals G1-G4 (those treated derived from embroys infected with the virus comprising the siRNA construct) showed markedly decreased expression of the lacZ gene as compared with untreated control animals.

WHAT IS CLAIMED IS:

1. A method of expressing an RNA molecule within a cell, the method comprising:

transfecting a packaging cell line with a retroviral construct;

recovering recombinant retrovirus from the packaging cell line; and

infecting a target cell with the recombinant retrovirus,

wherein the retroviral construct comprises the R and U5 sequences from a 5' lentiviral long terminal repeat (LTR), a self-inactivating lentiviral 3' LTR, an RNA Polymerase III promoter region and an RNA coding region operably linked to an RNA Polymerase III promoter region.

2. The method of claim 1, wherein the retroviral construct further comprises at least one termination sequence operably linked to the RNA coding region.

3. The method of claim 1, wherein the RNA Polymerase III promoter is inducible.

4. The method of claim 3, wherein the inducible promoter is activated with tetracycline.

5. The method of claim 1, wherein the RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region.

6. The method of claim 5, wherein the loop region is about 2 to about 10 nucleotides in length.

7. The method of claim 5, wherein the sense region and the antisense region are each between about 15 and about 30 nucleotides in length.

8. The method of claim 1, wherein the retroviral construct comprises a first RNA coding region operably linked to a first RNA Polymerase III promoter and a second RNA coding region operably linked to a second RNA Polymerase III promoter

9. The method of claim 8, wherein the first RNA coding region encodes a first RNA molecule and the second RNA coding region encodes a second RNA molecule.

10. The method of claim 9, wherein the first RNA molecule and the second RNA molecule are substantially complementary.

11. The method of claim 1, wherein the retroviral construct comprises a first RNA Polymerase III promoter and a second RNA Polymerase III promoter, each operably

linked to the RNA coding region, such that expression of the RNA coding region from the first RNA Polymerase III promoter results in the synthesis of a first RNA molecule and expression of the RNA coding region from the second RNA Polymerase III promoter results in the synthesis of a second RNA molecule substantially complementary to the first RNA molecule.

12. The method of claim 1, wherein expression of the RNA coding region results in the down regulation of a target gene, wherein the target gene comprises a sequence that is at least about 90% identical with the RNA coding region.

13. The method of claim 1 wherein said packaging cell line is a 293 cell line.

14. The method of claim 1 wherein the 5' LTR sequences are from HIV.

15. The method of claim 1, wherein the viral construct comprises the woodchuck hepatitis virus enhancer element sequence.

16. The method of claim 1, wherein the viral construct comprises a tRNA amber suppressor sequence.

17. The method of claim 1 wherein the self-inactivating 3' LTR comprises a U3 element with a deletion of its enhancer sequence.

18. The method of claim 17, wherein the self-inactivating 3' LTR is a modified HIV 3' LTR.

19. The method of claim 1, wherein the recombinant retrovirus is pseudotyped.

20. The method of claim 19, wherein the recombinant retrovirus is pseudotyped with the vesicular stomatitits virus envelope glycoprotein.

21. The method of claim 1, wherein the viral construct further comprises a gene of interest.

22. The method of claim 21, wherein the viral construct has a Polymerase II promoter operably linked to the gene of interest.

23. The method of claim 22, wherein the promoter is a CMV promoter.

24. The method of claim 22, wherein the viral construct additionally comprises an enhancer operably linked to the promoter.

25. The method of claim 25, wherein the enhancer and promoter are CMV sequences.

26. The method of claim 21, wherein the gene of interest is a reporter gene.

27. The method of claim 26, wherein the reporter gene encodes a fluorescent protein.

28. The method of claim 27, wherein the fluorescent protein is green fluorescent protein.

29. The method of claim 20, wherein the polymerase II promoter is a ubiquitous promoter.

30. The method of claim 27, wherein the ubiquitous promoter is selected from the group consisting of the ubiquitin promoter, the CMV β-actin promoter and the pgk promoter.

31. The method of claim 22, wherein the RNA Polymerase II promoter is a tissue specific promoter.

32. The method of claim 31, wherein said tissue specific promoter is selected from the group consisting of the lck promoter, the myogenin promoter and the thyl promoter.

33. The method of claim 1, wherein the target cell is an embryonic cell.

34. The method of claim 33, wherein the target cell is an embryogenic stem cell.

35. The method of claim 33, further comprising implanting the embryo in a pseudopregnant female.

36. The method of claim 1, wherein infecting a target cell comprises injecting the recombinant retrovirus between the zona pellucida and the cell membrane of a mammalian embryonic cell.

37. The method of claim 1, wherein infecting a target cell comprises removing the zona pellucida from a mammalian embryonic cell and incubating the cell in solution containing the recombinant retrovirus.

38. The method of claim 37, wherein the zona pellucida is removed by enzymatic digestion.

39. The method of claim 1, wherein the target cell is a cultured cell.

40. The method of claim 39, wherein the target cell is a cultured mammalian cell.

41. The method of claim 40, wherein the cultured mammalian cell is selected from the group consisting of CHO, HEK, COS and MEF cells.

42. The method of claim 1, wherein the target cell is an embryonic cell of a bird.

43. The method of claim 41, wherein infecting an embryonic cell of a bird comprises contacting the embryonic blastodisc of the bird egg with the recombinant retrovirus.

44. The method of claim 1, wherein the target cell is a fish egg.

45. The method of claim 42, wherein infecting the fish egg comprises delivering the recombinant retrovirus to the space between the chorion and the cell membrane of the fish egg.

46. A retroviral construct for the expression of an RNA molecule within a cell, the retroviral construct comprising:

a nucleic acid having the R and U5 sequences from a 5' lentiviral long terminal repeat (LTR);

a self-inactivating lentiviral 3' LTR; and

an RNA Polymerase III promoter.

47. The retroviral construct of claim 46, further comprising at least one termination sequence.

48. The retroviral construct of claim 46, wherein the RNA Polymerase III promoter is inducible.

49. The retroviral construct of claim 48, wherein the inducible promoter is activated with tetracycline.

50. The retroviral construct of claim 46, further comprising at least one RNA coding region operably linked to the RNA Polymerase III promoter.

51. The retroviral construct of claim 50, wherein the RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region.

52. The retroviral construct of claim 51, wherein the loop region is about 2 to about 10 nucleotides in length.

53. The retroviral construct of claim 51, wherein the sense region and the antisense region are between about 15 and about 30 nucleotides in length.

54. The retroviral construct of claim 50, wherein the RNA coding region encodes a first RNA molecule, and the retroviral construct further comprises a second RNA Polymerase III promoter and a second RNA coding region operably linked to the second

RNA Polymerase III promoter, wherein the second RNA coding region encodes a second RNA molecule substantially complementary to the first RNA molecule.

55. The retroviral construct of claim 50, wherein the retroviral construct further comprises a second RNA Polymerase III promoter operably linked to the RNA coding region, such that expression of the RNA coding region from the first RNA Polymerase III promoter results in a synthesis of a first RNA molecule and expression of the RNA coding region from the second RNA Polymerase III promoter results in synthesis of a second RNA molecule substantially complementary to the first RNA molecule.

56. The retroviral construct of claim 50, wherein expression of the RNA coding region results in the down regulation of a target gene,.

57. The method of claim 56, wherein the target gene comprises a sequence that is at least about 90% identical with the RNA coding region.

58. The retroviral construct of claim 46, wherein the 5' LTR sequences are from HIV.

59. The retroviral construct of claim 46, wherein the viral construct comprises the woodchuck hepatitis virus enhancer element sequence.

60. The retroviral construct of claim 46, wherein the viral construct comprises a tRNA amber suppressor sequence.

61. The retroviral construct of claim 46, wherein the self-inactivating 3' LTR comprises a U3 element with a deletion of its enhancer sequence.

62. The retroviral construct of claim 46, wherein the self-inactivating 3' LTR is a modified HIV 3' LTR.

63. The retroviral construct of claim 46, wherein the recombinant retrovirus is pseudotyped.

64. The retroviral construct of claim 63, wherein the recombinant retrovirus is pseudotyped with the vesicular stomatitits virus envelope glycoprotein.

65. The retroviral construct of claim 46, wherein the viral construct further comprises a gene of interest.

66. The retroviral construct of claim 65, wherein the viral construct has a Polymerase II promoter operably linked to the gene of interest.

67. The retroviral construct of claim 66, wherein the RNA Polymerase II promoter is a CMV promoter.

68. The retroviral construct of claim 66, wherein the viral construct additionally comprises an enhancer operably linked to the RNA Polymerase II promoter.

69. The retroviral construct of claim 68, wherein the enhancer and the RNA Polymerase II promoter are CMV sequences.

70. The retroviral construct of claim 66, wherein the RNA Polymerase II promoter is a ubiquitous promoter.

71. The retroviral construct of claim 70, wherein the ubiquitous promoter is selected from the group consisting of the ubiquitin promoter, the CMV β-actin promoter and the pgk promoter.

72. The retroviral construct of claim 66, wherein the RNA Polymerase II promoter is a tissue specific promoter.

73. The retroviral construct of claim 72, wherein said tissue specific promoter is selected from the group consisting of the lck promoter, the myogenin promoter and the thy1 promoter.

74. The retroviral construct of claim 65, wherein the gene of interest is a reporter gene.

75. The retroviral construct of claim 74, wherein the reporter gene encodes a fluorescent protein.

76. The retroviral construct of claim 75, wherein the fluorescent protein is green fluorescent protein.

FIGURE 1A

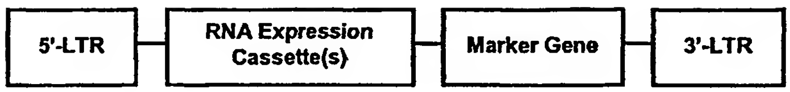

FIGURE 1B

FIGURE 2

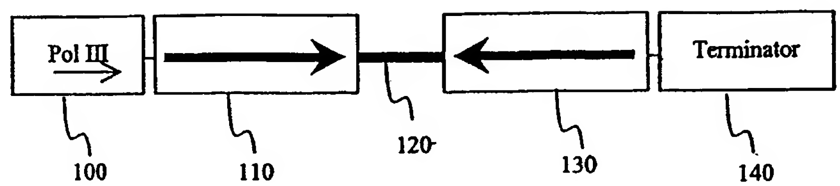

FIGURE 3

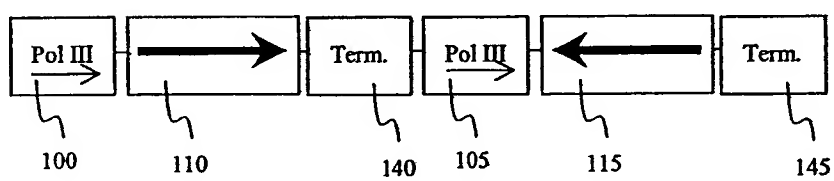

FIGURE 4

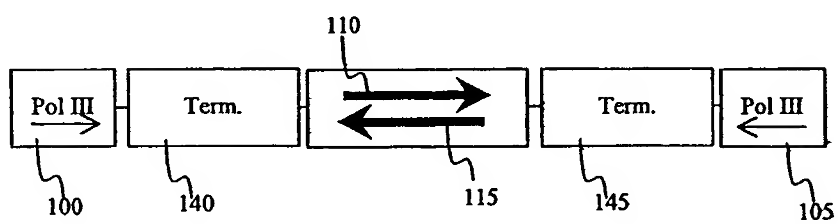

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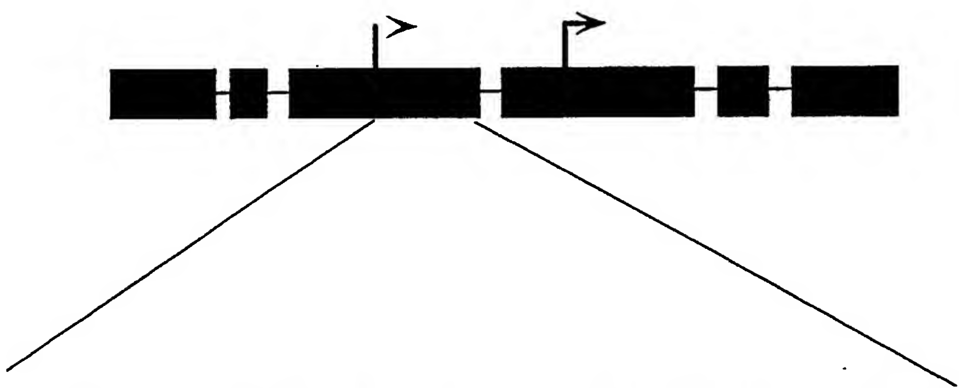
FIGURE 5



5' linker | Target (sense) | Loop | Target (antisense) | Terminator

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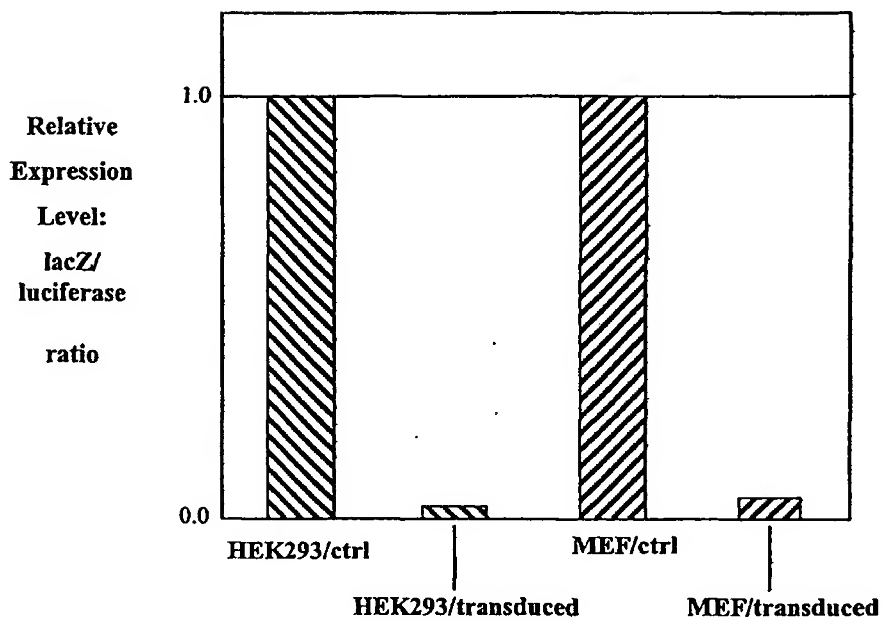
FIGURE 6


Relative
Expression
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luciferase
ratio
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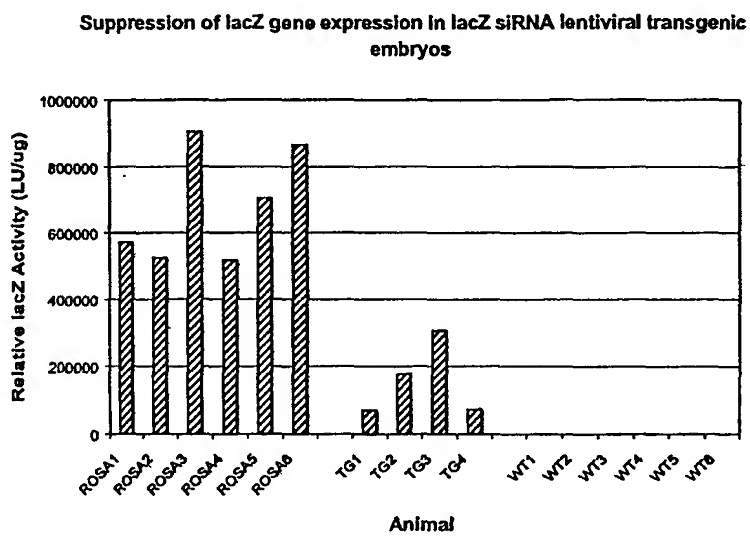
0.0
HEK293/ctrl
HEK293/transduced
MEF/ctrl
MEF/transduced

FIGURE 7


Suppression of lacZ gene expression in lacZ siRNA lentiviral transgenic embryos
Relative lacZ Activity (LU/ug)
1000000
800000
600000
400000
200000
0
ROSA1
ROSA2
ROSA3
ROSA4
ROSA5
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TG2
TG3
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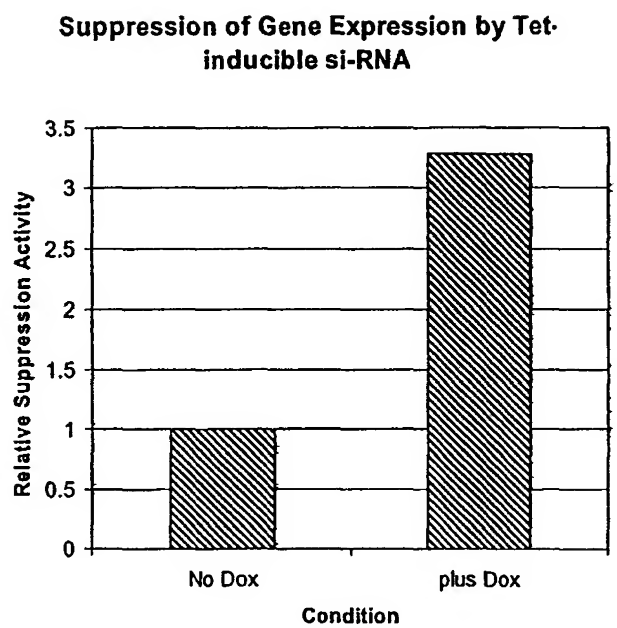
WT4
WT5
WT6
Animal

FIGURE 8

FIGURE 9



NUC 39124

SEQUENCE LISTING

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IN, XIAO-FENG
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BALTIMORE, DAVID

<120> METHOD FOR EXPRESSION OF SMALL RNA
MOLECULES WITHIN A CELL

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<150> 60/347,782
<151> 2002-01-09

<150> 60/389,592
<151> 2002-06-18

<150> Not yet available (Attorney Docket No.: CALTE.011PR)
<151> 2002-08-27

<160> 5

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<213> Artificial Sequence

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<210> 2
<211> 9941
<212> DNA
<213> Artificial Sequence

<220>
<223> This sequence represents a lentiviral vector
comprising a human immunodeficiency virus flap
sequence, a green fluorescent protein variant
sequence, a human ubiquitin promoter sequence and
a woodchuck hepatitis regulator element sequence.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/29215

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01N 43/04; C07H 21/02, 21/04; C12N 15/00
US CL : 514/44; 536/23.1; 536/24.5; 435/320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 536/23.1; 536/24.5; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, STN-Medline Biosis EMBASE Lifesci CAplus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,096,538 A (KINGSMAN et al) 1 August 2000 (01.08.2000), column 2, lines 6-12; column 7, lines 41-43; column 3, lines 57-67, Figure 2B.	1-3,8,9,17,21, 22, 29, 46-48, 50, 61, 62, 66, 70,74
Y	US 5,883,081 A (KRAUS et al) 16 March 1999 (16.03.1991), column 29, lines 15-60; column 47, Example 10; column 43, lines 65-67); column 48 lines 49-50; column 47, lines 65-66 and Table 2.	1,14,22,23,29,30, 39-41,19,20, 46,63,64,66,67,70,71
Y	REISER et al. Development of Multigene and Regulated Lentivirus Vectors. Jour. Virol. November 2000, Vol. 74, No. 22, pages 10589-10599.	1-4, 19-30,39,40,46-50, 63-71,74-76
Y	IWAKUMA et al. Self-inactivating Lentiviral Vectors with U3 and U5 Modifications. Virology, 1999, Vol. 261, pages 120-132.	1,17,18,46,61,62
Y	GATLIN et al. Long-term engraftment of nonobese diabetic/severe combined immunodeficient mice with human CD34+ cells transduced by a self-inactivating human immunodeficiency virus type I vector. Human Gene Therapy, 10 June 2001, Vol. 12, pages 1079-1089.	1,17-18,46,61,62
Y	US 6,255,071 B1 (BEACH et al) 03 July 2001 (03.07.2001), col. 6, lines 43-47; col. 13, lines 60-65; col. 14, lines 35-36	1,2,13,46

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2003 (15.01.2003)	10 FEB 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Felicia D. Roberts for Valarie Bertoglio Telephone No. 703-308-1234

Form PCT/ISA/210 (second sheet) (July 1998)

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C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GODWIN et al. Detection of targeted GFP-Hox gene fusions during mouse embryogenisis. PNAS, October 1998, Vol. 95, pages 13042-13047, specificall Abstract and page 13042, col. 2, "Vector Construction".	27,28,75,76
Y	TIMMONS et al. Specific interference by ingested dsRNA. Nature, 29 October 1998, Vol. 395, page 854, see whole document.	11,15
Y	ILVES et al. Retroviral Vectors designed for targeted expression of RNA polymerase III-driven transcripts: a comparative study. Gene, 1996, Vol. 171, pages 203-208, specifically page 203 Abstract and Introduction; pages 207, column 1, lines 14-17.	1,8-11,46,54,55
Y	JUNKER et al. Reduction in replication of the human immunodeficiency virus type I in human T cell lines by polymerase III-driven transcription of chimeric tRNA-antisense RNA genes. Antisense Research and Development, 1994, Vol. 4, pages 165-172, specifically page 166, column 1, lines 22-23.	1,8-11,46,54,55
Y	OGUETA et al. Design and in vitro characterization of a single regulatory module for efficient control of gene expression in both plasmid DNA and a self-inactivating lentiviral vector. Molecular Medicine, 2001, Vol. 7, No. 8, pages 569-579.	1,3,4,46,48,49,61,62
Y,P	BRUMMELKAMP et al. A system for stable expression of short interfering RNAs in mammalian cells. 19 April 2002, Vol. 296, pages 550-553, specifically column 3, lines 2-3 and 12-19.	1,5-11,46,54,55
Y	KAFRI et al. Lentiviral Vectgors: Regulated gene expression. Molecular Therapy, June 2000, Vol. 1, No. 6, pages 516-521.	4

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/027298 A1

(54) Title: STEM-LOOP VECTOR SYSTEM

(57) Abstract: The present invention relates to a vector system for use in the generation of nucleic-acid libraries and in methods of cosuppression. The invention resides in the use of a vector comprising a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion comprising at least one unique restriction endonuclease site.

STEM-LOOP VECTOR SYSTEM

FIELD OF THE INVENTION

The present invention relates generally to a method for generating a nucleic acid library. More particularly, the present invention provides a library of eukaryotic-derived nucleic acid molecules inserted into vectors and maintained in a prokaryotic microorganism or as isolated and/or purified nucleic acid molecules. Such molecules are useful for transforming or otherwise being introduced to eukaryotic cells which can then be screened for transcriptional or post-transcriptional gene silencing (TGS or PTGS) events.

BACKGROUND OF THE INVENTION

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

The increasing sophistication of recombinant DNA techniques has provided significant progress in understanding the mechanisms regulating eukaryotic gene expression. This is greatly facilitating research and development in the plant, agricultural, medical and veterinary industries. One important aspect is the development of means to alter the phenotype of a cell or group of cells by modulating the expression of genetic material. A myriad of desirable phenotypic traits is potentially obtainable following selective inactivation of gene expression. However, whilst advances have been made in regulating gene expression, much less progress has been made in the actual manipulation of gene expression to produce such novel traits. Moreover, only limited means, by which human intervention may lead to a modulation of the level of eukaryotic gene expression, have been available.

In the literature, the term "gene silencing" is frequently used. This has generally been done, however, in the absence of an appreciation of whether the gene silencing events were acting in *cis* or in *trans*. This is relevant to the commercial exploitation of gene silencing technology, since *cis* inactivation events are of less usefulness than events in *trans*. For example, there is less likelihood of success in targeting endogenous genes (e.g. plant genes) or exogenous genes (e.g. genes from pathogens) using techniques that require *cis* inactivation.

One approach to gene inactivation (i.e. the inactivation of gene expression) utilizes anti-sense nucleic acid molecules directed to complementary mRNA transcripts. It has been postulated that a double-stranded mRNA forms by base pairing between the complementary nucleotide sequences to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

In an alternative approach, the expression of an endogenous gene in a cell, tissue or organ may be suppressed when one or more copies of the gene, or one or more copies of a substantially similar gene, are introduced into the cell. The transgene is expressed as sense RNA. This appears to involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve either repression at the level of transcription, in which somatically-heritable repressed states of chromatin are formed or alternatively, repression following transcription, in which case transcription initiation occurs normally but RNA products are subsequently eliminated. In other words, gene inactivation may occur in *cis* or in *trans*. For *cis* inactivation, only the target gene is inactivated and other similar genes dispersed throughout the genome are not affected. In contrast, inactivation in *trans* occurs when one or more genes dispersed throughout the genome and sharing homology with a particular target sequence are also inactivated.

The term "co-suppression" is used to describe the latter form of PTGS. Expression of such transgene sequences results in inactivation of homologous genes, i.e. a sequence specific in *trans* inactivation of gene expression (Napoli *et al., The Plant Cell 4:* 279-289, 1990; van

der Krol *et al., The Plant Cell 4:* 291-299 1990). The molecular phenotype of cells in which this occurs is well described in plant systems and the disappearance of mRNA sequences is thought to occur as a consequence of activation of a sequence-specific RNA degradative system (Lindbo *et al., The Plant Cell 5:* 1749-1759, 1993; Waterhouse *et al., Proc. Natl. Acad. Sci. USA 95:* 13959-13964, 1999).

In essence, co-suppression is thought to involve the generation of interference RNA (RNAi). RNAi refers to PTGS induced by double-stranded RNA (dsRNA). It has been shown that injection of dsRNA into *C. elegans* leads to sequence-specific gene silencing (Fire *et al., Nature 391:* 806-811, 1998). Ingestion of dsRNA (Timmons and Fire, *Nature 395*: 854, 1998) or bacteria expressing gene constructs to produce dsRNA (Timmons *et al., Gene 263:* 103-112, 2001) also leads to PTGS. RNAi has since been demonstrated to be effective in a range of organisms including *Drosophila* (Caplen *et al., Gene 252:* 95-105, 2000; Fortler and Belote, *Genesis 264:* 240-244, 2000), spiders (Schoppmeier and Damen, *Development Genes & Evolution 211:* 76-82, 2001) and mammals (Elbashir *et al., Nature 411:* 494-498, 2001).

The frequency of PTGS induced by transgene expression can be increased by use of hairpin or inverted repeat (IR) gene constructs (Singh *et al., Biochemcial Society Transactions 28:* 925-927, 2000; Smith *et al., Nature 407:* 319-320, 2000). Such constructs have been shown to produce close to 100% PTGS frequencies in plants. Inverted repeat constructs are also effective in animals, for example, *Drosophila* (Fortier and Belote, 2000, *supra*) and *C. elegans* (Timmons *et al.*, 2001, *supra*). However, creation of an IR construct can only be achieved one gene at a time and requires multiple cloning steps. Accordingly, current methods for generating IR gene constructs are time consuming and labour intensive. There are no known methods for creating a library of inverted repeat or hairpin gene constructs in a single cloning step.

U.S. Patent No. 6,054,299 describes a method for constructing a stem-loop cloning vector. The vector is useful for producing a single-stranded nucleic acid molecule that is to be *cis*-activated by a desired double-stranded genetic element, for example, a promoter. The

nucleic acid molecule is cloned into a double-stranded replicative form of the vector between a pair of IR sequences. The IR sequences encode the double-stranded genetic element. When expressed as a single-stranded DNA, the cloned nucleic acid is located in a single-stranded "loop" region of a "stem-loop" structure. U.S. Patent No. 6,054,299 does not describe or allude to cloning of a DNA fragment into the double-stranded stem of the "single-stranded" form of the vector, nor does the disclosure provide a means for cloning a double-stranded nucleic acid to create an inverted repeat.

There has been considerable confusion within the animal literature regarding the term "co-suppression" (Bingham, *Cell 90:* 385-387, 1997). In fact, until relatively recently, "co-suppression", as defined by the specific molecular phenotype of gene transcription without translation, was considered not to occur in mammalian systems. It had been described only in plant systems and in a lower eukaryote, *Neurospera* (Cogoni *et al., EMBO J. 15:* 3153-3163, 1996; Cogoni & Mancino, *Proc. Natl. Acad. Sci. USA 94:* 10233-10238, 1997). However, work over the past few years has shown that comparable post-transcriptional inactivation events do, in fact, occur in mammalian and other animal systems.

A range of different genetic constructs has been demonstrated to be efficacious in systems designed to down-regulate or otherwise modulate gene expression in either plant or animal cells.

Until now, however, use of such constructs in relation to a target endogenous sequence has relied on generating particular sequence-specific genetic molecules designed to interfere with the expression of a known target sequence, requiring that both the particular nucleic acid sequence and the biological function of a targeted endogenous gene be known. Furthermore, the manufacture of such constructs requires multiple cloning steps and is generally done on a gene-by-gene (i.e. trait-by-trait) basis, addressing only one trait/phenotype at a time and making the entire process extremely labour-intensive.

With the advent of sophisticated means of high throughput screening and micro-array technologies, very large numbers of molecules are able to be generated and screened for

desired characteristics simultaneously and rapidly, thereby greatly increasing the efficiency of commercially-directed research protocols and product development. For the types of gene-modulating constructs described above to be routinely applied in diagnostically and therapeutically useful ways, there is a need for the creation of much more rapid and predictable means of generating libraries of potentially functionally relevant genetic molecules.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc.

In work leading up to the present invention, the inventor has developed a vector system useful in the generation of nucleic acid libraries comprising eukaryotic-derived genetic sequences. These sequences may comprise cDNA and/or genomic DNA. The genomic DNA may comprise one or more promoter or other regulatory or non-transcribed regions. When introduced into particular eukaryotic cells, the nucleic acid molecules in the library may generate partially double-stranded RNA transcripts. The library is useful, therefore, for producing nucleic acid molecules that result in gene silencing in eukaryotic cells. The RNA transcripts of the present invention are referred to herein as "co-suppression effectors". They may take the form of a "hairpin-shaped construct" or a "perfect hairpin", as described hereinafter. Silencing may occur *via* PTGS, where the library comprises genetic sequences derived from, for example, cDNA or genomic DNA corresponding to an amino acid-encoding or RNA-encoding region of a genetic sequence. Alternatively, it may occur *via* TGS, where the library comprises genetic sequences derived from, for example a non-transcribed promoter or other regulatory DNA region. In this second case, the library may generate partially double-stranded RNA transcripts targeted at, for example, a non-transcribed promoter region, resulting eventually in TGS, such as *via* DNA methylation.

The present invention provides, therefore, a range of genetic molecules referred to herein respectively as a co-suppression vector, co-suppression constructs (in double-stranded and partially single-stranded forms), a co-suppression library and co-suppression effectors.

The co-suppression vector may comprise a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded DNA portion comprising at least one restriction endonuclease site. This is referred to herein as a "co-suppression vector". When maintained in a prokaryotic organism, in the absence of helper phage, the vector may be in a double-stranded form.

Into the double-stranded portion of the co-suppression vector may be introduced eukaryotic DNA. The eukaryotic DNA may comprise cDNA or genomic DNA. This then becomes the partially single-stranded form of the co-suppression construct and is referred to herein as "ss co-suppression construct" or "co-suppression construct (i)". The resulting recombinant molecule may be introduced into, for example, a prokaryotic microorganism to produce a library of double-stranded co-suppression constructs comprising the eukaryotic DNA. This is the double-stranded form of the co-suppression construct and is referred to herein as "ds co-suppression construct" or "co-suppression construct (ii)".

The co-suppression constructs of the invention are generated from a double-stranded DNA cloning vector, according to the method described herein.

In one particular embodiment, the present invention may be described as including the following steps:

(i) conversion of a double-stranded replicative circular DNA cloning vector, comprising an inverted repeat sequence, into a single-stranded form;

(ii) treatment of said single-stranded form such that self-complementary sequences derived from the inverted repeat (IR) sequence anneal to form a region of double-stranded nucleic acid;

(iii) cleavage of the double-stranded region formed in step (ii) by one or more restriction enzymes to form a vector stem-loop portion and a spacer stem-loop

portion;

(iv) ligation of (iii) with double-stranded DNA fragments containing termini compatible with the vector and spacer stem-loops;

(v) conversion of recombinant nucleic acid molecules derived from step (iv) into double-stranded circular form, thereby generating a nucleic acid construct containing an IR of the cloned double-stranded fragment(s), referred to herein as a co-suppression construct or an IR DNA construct.

Preferably, the double-stranded region formed in step (ii) contains at least one restriction enzyme recognition site and is of sufficient length to stabilise the stem/loop structures formed by subsequent cleavage in step (iii).

Where two restriction enzymes are used in step (iii), a double-stranded linear fragment will be concomitantly released. Most restriction enzymes only cleave double-stranded DNA. Hence, cleavage in step (iii) should only occur in the annealed double-stranded region, not in other single-stranded regions of the vector, even if there are additional restriction endonuclease recognition sites in the single-stranded loop regions.

The conversion of recombinant nucleic acid molecules into double-stranded form, effected in step (v), can be achieved either *in vitro* or by transformation of a host cell which will convert it to double-stranded form as part of the replicative process.

The double-stranded DNA cloning vector, with which the method of the present invention begins, may comprise one or more promoters operable in eukaryotic cells. A co-suppression construct generated therefrom may, therefore, also comprise one or more promoters operable in eukaryotic cells and operably linked to a portion of the co-suppression vector upstream of the IRs.

Consequently, upon introduction of the co-suppression library into a suitable eukaryotic

cell, expression mediated by a eukaryotic promoter results in double-stranded eukaryotic RNA with or without a stem loop, depending on whether or not the spacer is an intron. The double-stranded eukaryotic RNA with or without a stem loop is referred to herein as a "co-suppression effector".

Eukaryotic cells carrying the co-suppression effector RNA are then screened for the effects of PTGS or TGS.

In an alternative embodiment, the double-stranded DNA cloning vector from which the co-suppression constructs of the invention are generated, may comprise one or more promoters operable in prokaryotic cells. In this case, a co-suppression construct generated therefrom may, therefore, also comprise one or more promoters operable in prokaryotic cells and operably linked to a portion of the co-suppression vector upstream of the IRs.

Accordingly, when eukaryotic DNA is introduced into the double-stranded portion of the partially single-stranded vector, the resulting recombinant molecule, when introduced into a prokaryotic microorganism, produces a double-stranded co-suppression library comprising the introduced eukaryotic and prokaryotic DNA. One or more promoters operable in prokaryotic cells are comprised within the introduced DNA. Expression of this form of the co-suppression library in a prokaryotic cell is then mediated by a prokaryotic promoter, again resulting in co-suppression effectors. This form of the co-suppression library may be used in feeding situations, where the ingestion of the library by a eukaryotic organism may result in the generation of co-suppression effector RNAs which interact with the nucleic acid material of the eukaryotic organism, possibly resulting in PTGS.

In either embodiment, the co-suppression effector RNAs may cause gene silencing, either *via* PTGS or *via* TGS, depending on the identity of the genetic sequences comprised in the co-suppression construct from which the co-suppression effector RNA was derived.

The present invention provides, therefore, a co-suppression library either in prokaryotic microorganisms or as nucleic acid molecules in an isolated or purified form. The co-

suppression library comprises eukaryotic DNA, generally randomly generated by digestion of a particular eukaryotic genome. The co-suppression library may also further comprise prokaryotic DNA. The generation of the library does not require any prior knowledge of a target gene. All that is required is an appropriate eukaryotic indicator cell line. Such a cell line is used to identify TGS or PTGS *via* a detectable trait or reporter signal.

The present invention further provides isolated or purified prokaryotic cells comprising the co-suppression library of double-stranded co-suppression constructs, single-stranded co-suppression constructs or co-suppression vectors. The present invention further provides eukaryotic or prokaryotic cells comprising co-suppression effectors.

A summary of sequence identifiers and a glossary of important terms used throughout the subject specification are provided in Tables 1 and 2, respectively.

TABLE 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	*Acc*65I
2	*Bsi*WI
3	PpoAcc primer
4	PpoBsi primer
5	PPO-Srf-F primer
6	PPO-Srf-R primer
7	MCS-Sac-Dra
8	MCS-Kpn-Mlu

TABLE 2

Glossary of terms

TERM	MEANING
double-stranded DNA cloning vector	DNA vehicle comprising or into which has been cloned particular useful features such as, for example, a selectable marker gene, inverted repeat sequences, multiple cloning sites, prokaryotic and/or eukaryotic promoter regions, stop codon, *inter alia*
co-suppression vector	single-stranded form of the DNA cloning vector, having a double-stranded stem portion into which eukaryotic DNA may be inserted
co-suppression construct (i)	a co-suppression vector comprising eukaryotic DNA
co-suppression construct (ii)	the double-stranded form of a co-suppression construct (i)
co-suppression library	a mixture of co-suppression constructs (ii), either in a prokaryotic organism or in isolated form
co-suppression effector	an RNA molecule transcribed from co-suppression construct (ii)

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of the protocol for generating a library of eukaryotic nucleic acid molecules (referred to herein as co-suppression constructs and co-suppression effectors) in prokaryotic cells and testing these in eukaryotic cells.

Figure 2 is a diagrammatic representation showing a production of a single-stranded co-suppression construct by a single cloning step. A double-stranded DNA cloning vector (refer to Figure 7) is converted into a predominantly single-stranded form and a self-complementary inverted repeat (IR) region is allowed to anneal to form a double-stranded region. One or more restriction enzyme recognition sites within the double-stranded region is then cut with an appropriate enzyme(s), producing two stem-and-loop structures. Ligation of a stem-and-loop with a compatible double-stranded DNA fragment produces a double-stranded region flanked by single-stranded loops.

Figure 3 is a diagrammatic representation showing one end of a double-stranded nucleic acid fragment (e.g. heterologous nucleic acid) that is ligated to a compatible stem-and-loop nucleic acid to form a stem-and-loop DNA molecule (referred to herein as a co-suppression construct). The construct is converted to a double-stranded form by synthesis of the complementary strand thereby creating a spacer DNA region flanked by an IR of the original double-stranded DNA polynucleotide. Synthesis of the complementary strand may be achieved either *in vitro* using, for example, a DNA polymerase and a suitable primer or *in vivo* using, for example, the DNA replication mechanism provided by a host cell.

Figure 4 is a diagrammatic representation showing conversion of the nucleic acid shown in Figure 2 to a double-stranded form by synthesis of a complementary strand to produce a double-stranded co-suppression construct comprising a spacer region flanked by IRs of the cloned DNA fragment.

Figure 5 is a diagrammatic representation showing a single-stranded co-suppression vector, comprising a short IR that does not require an intervening spacer region to enable

replication in bacteria. This single-stranded vector can be cut with appropriate restriction enzyme(s) to produce a plasmid (replicon)+stem portion, which can subsequently be either self-ligated or ligated with a compatible DNA fragment comprising a spacer+-stem portion.

Figure 6 is a diagrammatic representation showing production of a stem-and-loop structure *via* polymerase chain reaction (PCR). PCR amplification of DNA fragments with two primers comprising regions of sequence identity results in creation of amplification products comprising IRs at their termini. PCR amplification of one strand, using a single primer results in production of predominantly single-stranded products, which can self-anneal to form stem-and-loop structures. These structures can then be first digested with a restriction enzyme or directly ligated with compatible fragments for the creation of co-suppression constructs.

Figure 7 is a diagrammatic representation showing an example of cloning steps that can be used to produce a double-stranded DNA cloning vector.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMDENTS

The present invention is predicated in part on the development of a vector useful for generating a nucleic acid library of eukaryotic genetic sequences and/or a combination of eukaryotic and prokaryotic genetic sequences, in double-stranded DNA form. The vector is referred to herein as a "co-suppression vector". The library of eukaryotic DNA inserts is referred to herein as a "co-suppression library". Any individual co-suppression vector comprising a eukaryotic DNA insert is referred to herein as co-suppression construct. Such co-suppression constructs may, alternatively, be referred to as inverted repeat (IR) DNA constructs. Conveniently, the co-suppression library is maintained in a prokaryotic cell. The present invention extends, however, to the co-suppression library in isolated and/or purified form.

The co-suppression vector permits the generation of a co-suppression library of eukaryotic genetic sequences. Introduction of a particular co-suppression library to eukaryotic cells, followed by expression, results in RNA having a double-stranded portion. The ability of the co-suppression library to introduce or otherwise facilitate gene silencing of particular eukaryotic genes can then be screened for. No knowledge of the eukaryotic sequences is required. The library, in one form, comprises randomly generated representatives of a eukaryotic genome. When introduced into a particular eukaryotic cell line, PTGS or TGS is monitored by, for example, alteration of a particular trait or change in a particular signal.

Accordingly, in one embodiment, the present invention contemplates a method for generating a library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a single-stranded replicon portion and a single-stranded loop portion separated by double-stranded stem portion comprising at least one restriction endonuclease site;

(ii) digesting said partially single-stranded vector with at least one restriction

endonuclease and admixing therewith double-stranded genomic DNA or cDNA derived from a eukaryotic cell and digested with the same restriction endonuclease or other enzyme or under conditions providing compatible 3' and 5' end portions for ligation into the restricted partially single-stranded vector and subjecting said admixture to ligation conditions to generate the partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) introducing the ligated admixture of (ii) into said suitable cell under conditions to permit the generation of a double-stranded replicative form of said partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments.

In an alternative embodiment, the double-stranded replicative form of said partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments is first generated *in vitro* from the ligated admixture. The replicative form is then subsequently introduced into a suitable cell.

Suitable cells comprise eukaryotic cells and prokaryotic microorganisms.

Accordingly, in another embodiment, the present invention contemplates a method for generating a library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a single-stranded replicon portion and a single-stranded loop portion separated by double-stranded stem portion comprising at least one restriction endonuclease site;

(ii) digesting said partially single-stranded vector with the at least one restriction endonuclease and admixing therewith double-stranded genomic DNA or cDNA derived from a eukaryotic cell and digested with the same restriction endonuclease or other enzyme or under conditions providing compatible 3' and 5' end portions

for ligation into the restricted partially single-stranded vector and subjecting said admixture to ligation conditions to generate the partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) generating, *in vitro*, a double-stranded replicative form of said partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments from the ligated admixture of (ii), prior to introducing the resulting double-stranded replicative form into said suitable cell.

The double-stranded stem portion of step (i) arises from self-annealing of complementary sequences derived from the IR sequences introduced into a double-stranded DNA cloning vector. Generation and subsequent use of DNA cloning vectors is described hereinafter.

The partially single-stranded vector may be digested with a single restriction endonuclease or a combination of two or more restriction endonucleases (step (ii)).

The resulting double-stranded replicative form generated by step (iii) comprises exogenous eukaryotic DNA and is referred to herein as a double-stranded co-suppression construct. The population of molecules represents the co-suppression library. The co-suppression library may be isolated or purified nucleic acid molecules, or may be a culture of cells comprising same.

In one preferred embodiment, the culture comprises a prokaryotic microorganism.

Any suitable prokaryotic microorganism may be utilized as a host for the library of nucleic acid molecules generated by the above method, the requirement being that when a partially single-stranded form of the co-suppression vector is required, the microorganism has to support formation of a single-stranded replicative form, generally with the use of a helper phage. At other times, any other prokaryotic microorganism may be employed.

The generation of a partially single-stranded vector having a single-stranded replicon portion and a single-stranded spacer-loop portion separated by a double-stranded stem comprising at least one restriction endonuclease site (step (i)) is initiated by first obtaining a double-stranded DNA cloning vector having a multiple cloning site. A "multiple cloning site" means a multiplicity of two or more restriction endonuclease sites and preferably one or more unique restriction endonuclease sites which, upon digestion, results in a vector being cleaved only within the multiple cloning site.

A range of vectors may be employed at this step but a pBluescript vector is particularly useful. One particular pBluescript vector comprises the multiple cloning site comprising the restriction endonuclease sites *Bss*HI, *Acc*651, *Apa*I, *Xho*I, *Sal*I, *Eco*RI, *Not*I and *Sac*I. The multiple cloning site is flanked by *Bss*HI sites.

A spacer nucleic acid molecule is then cloned into the multiple cloning site. Preferably, but certainly not exclusively, the spacer molecule is or comprises an intron.

An intron is useful in that upon expression in a eukaryotic cell, the intronic spacer is spliced out from the transcript. A spacer may be regarded as a "hinge" to permit homologous nucleotide sequences on two strands separated by the hinge to fold back and anneal to each other. Preferably, the length of the spacer is such as to not adversely impact on the efficiency of self-annealing of the complementary homologous sequences.

The PPO intron from the pineapple PPO gene is particularly useful as a spacer element although any intron may be employed.

The spacer is inserted into the multiple cloning site leaving, generally but not exclusively, one or more unique restriction sites adjacent both sides of the spacer. If more than one restriction site and, hence, restriction endonuclease, is used, directional cloning of digested DNA fragments is facilitated. Moreover, a stretch of the double-stranded stem may be able to be removed, and inserts of predetermined length - such as may be generated following size fractionation - may be cloned therein.

The double-stranded cloning vector being constructed also requires homologous nucleotide sequences flanking the spacer. The multiple cloning site is regarded as the first of these nucleotide sequences. The introduction of the same or a homologous multiple cloning site, in inverse orientation, on the other side of the spacer to the one within or adjacent the first multiple cloning site, is required. The spacer is preferably inserted into one end of the multiple cloning site. To achieve this, the next step in constructing the cloning vector is to digest it with appropriate restriction endonuclease enzymes, to isolate a fragment comprising the multiple cloning site or part thereof and the spacer. The same or homologous multiple cloning site is then excised from another vector and a three-way ligation reaction initiated with a cloning vector so as to produce a cloning vector having two IRs in the form of the same or homologous multiple cloning sites flanking the spacer. For a diagrammatic representation of this procedure, refer to Figure 7.

There are a number of different ways in which the double-stranded DNA cloning vector may be generated and the present invention is not to be limited to any one means of production.

Accordingly, another aspect of the present invention contemplates a method for generating a double-stranded DNA cloning vector useful for generating a co-suppression library, said method comprising introducing into a double-stranded vector, which is optionally capable of generating single-stranded replicative intermediates in the presence of a helper phage, two homologous nucleotide sequences flanking a spacer nucleotide sequence such that, when in single-stranded form, the spacer nucleotide sequence permits the two homologous nucleotide sequences to anneal together to create a partially double-stranded molecule.

In one embodiment, the double-stranded DNA cloning vector comprises some but not all the genetic material required to replicate *via* a single-stranded intermediate. Consequently, in the presence of a helper phage in a prokaryotic microorganism, a single-stranded replicative intermediate is generated. This is a co-suppression vector.

Following standard biochemical extraction and precipitation techniques, the co-suppression vector is isolated. *In vitro*, the single-stranded co-suppression vector may comprise a double-stranded portion comprising the two same or homologous multiple cloning sites. The "replicon portion" of the co-suppression vector is derived from the double-stranded DNA cloning vector initially employed and permits replication in a host microbial microorganism. The spacer "loop portion" comprises the nucleic acid spacer sequence which was cloned into, and hence separated, the multiple cloning site, and the "double-stranded stem portion" comprises the one or more restriction endonuclease recognition sequences from two of the same or homologous multiple cloning sites.

These restriction endonuclease recognition sequences may then serve as cloning sites, using standard cloning procedures well known in the art. Any restricted double-stranded genomic DNA or cDNA preparation may be ligated therein, provided that it has compatible 3' and 5' ends. Both sticky ends and ends that have been appropriately blunted may be comprised within the term "compatible". Reference to a "cDNA" includes cDNA corresponding to a single gene as well as to two or more genes from the genome of an organism.

In an alternative embodiment, a co-suppression construct may be generated *in vitro* by "nicking" of one strand of a double-stranded DNA cloning vector. The nicked strand is then digested with, for example, Exonuclease III, to leave a single-stranded circular DNA remaining. Upon exposure to annealing conditions, a self-complementary complex forms, comprising two single-stranded "loop portions" joined by a double-stranded "stem". Digestion with a suitable enzyme(s) produces a "replicon stem-loop" and a "spacer stem-loop", which may again be ligated with compatible eukaryotic DNA.

In one preferred embodiment, restriction endonucleases having non-palindromic recognition sequences are utilised. With such enzymes it is possible to reduce the background that is sometimes observed due to, for example, ligation of replicon stem-loops to each other.

For the construction of a library of co-suppression constructs according to the methods of the present invention, a genomic or cDNA preparation from any eukaryotic organism may be fragmented. The choice of eukaryotic organism will be determined only by the species and target of interest. Suitable eukaryotic cells include *inter alia* those derived from plants as well as animals, such as mouse and livestock animals as well as human animals and invertebrate animals such as insects and nematodes.

Accordingly, another aspect of the present invention provides a co-suppression construct comprising two single-stranded DNA loop portions separated by a double-stranded portion wherein the double-stranded portion comprises one or more restriction endonuclease sites into which has been introduced a double-stranded DNA fragment.

This is the partially single-stranded form of the co-suppression construct, also referred to as co-suppression construct (i). In a suitable cell such as, for example, a prokaryotic microorganism, this co-suppression construct is converted into a double-stranded form, referred to as a co-suppression construct (ii) or, alternatively, an IR DNA construct. The present invention extends to a mixture of single-stranded and double-stranded forms as may exist in, for example, M13. As a result of carrying out the methods of the present invention, a library of such nucleic acid molecules may be generated.

A co-suppression construct may also be replicated *in vitro*, such as *via* rolling circle replication, to generate a concatamer comprising multiple copies of one strand of a co-suppression construct. Upon exposure to annealing conditions, a nucleic acid complex forms, comprising multiple "stem-loop" portions. Digestion with a suitable enzyme provides a source of spacer-stem portions for ligation to compatibly digested replicon-stem portion of a co-suppression construct. Thereby hybrid co-suppression constructs may be generated.

Stem-loop structures may also be generated *via* PCR amplification. Amplification products may be ligated to blunt-ended double-stranded DNA fragments and/or to replicon-stem portions to form a co-suppression construct. Alternatively, a single-stranded spacer-loop

with self-complementary ends may be ligated to a single-stranded cDNA polynucleotide and used to prime second strand synthesis. Double-stranded stem portions thereby generated may be ligated to double-stranded DNA fragments and/or to replicon-stem portions to form a co-suppression construct.

Hence, a co-suppression construct may be generated by any number of means and may be in any number of forms. Preferably, expression of the double-stranded form results in the formation of RNA comprising either a stem-loop in the form of a hairpin, or a perfect hairpin. These are referred to herein as co-suppression effectors.

A co-suppression effector in the form of a "hairpin-shaped" comprises the spacer nucleotide sequence flanked on each side by an IR sequence capable of annealing to form a double-stranded portion. Where such annealing occurs, the co-suppression effector takes the form of a double-stranded portion and a loop portion, thus resembling a hairpin in shape.

As mentioned above, the spacer loop portion of a co-suppression construct or vector need not necessarily comprise an intron. However, where it does, subsequent transcription and restriction of the double-stranded form thereof results in the creation of RNA molecules which comprise a double-stranded nucleotide sequence, from which the single-stranded loop, which would have been an intron, has been spliced out. The co-suppression effector RNA molecule thus formed is referred to herein as a "perfect" hairpin, inasmuch as the stem-loop structure of the "hairpin" has been altered to yield only the double-stranded stem portion.

In another embodiment, the present invention provides a mixture of nucleic acid co-suppression effector molecules in the form of double-stranded RNA optionally with a single-stranded loop portion, formed by *in vitro* transcription and/or processing of a co-suppression construct.

Accordingly, another aspect of the present invention contemplates a method for generating

a co-suppression library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a co-suppression vector having a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion comprising at least one restriction endonuclease site;

(ii) digesting the double-stranded portion of the vector with the at least one restriction endonuclease and admixing therewith a double-stranded genomic DNA or cDNA preparation digested with the same restriction endonuclease or with an enzyme or under conditions providing compatible 3' and 5' end portions for ligation into the restricted co-suppression vector and subjecting said admixture to ligation conditions to generate the co-suppression construct comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) introducing the ligated admixture of (ii) into said suitable cell under conditions to permit the generation of a double-stranded replicative form of said co-suppression construct.

Reference herein to a viral-derived nucleic acid molecule includes reference to nucleic acid molecules derived from a virus such as but not limited to a geminivirus or other plant virus, a retrovirus, a human immuno-deficiency virus or a hepatitis virus, *inter alia*.

In an alternative embodiment, the double-stranded replicative form of the co-suppression construct is first generated *in vitro* from the ligated admixture. The replicative form is then subsequently introduced into a suitable cell.

Accordingly, in another embodiment, the present invention contemplates a method for generating a library of eukaryotic-derived nucleic acid molecules in a suitable cell, said

method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a co-suppression vector having a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion comprising at least one restriction endonuclease site;

(ii) digesting the double-stranded portion of the vector with the at least one restriction endonuclease and admixing therewith a double-stranded genomic DNA or cDNA preparation digested with the same restriction endonuclease or with an enzyme or under conditions providing compatible 3' and 5' end portions for ligation into the restricted co-suppression vector and subjecting said admixture to ligation conditions to generate the co-suppression construct comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) generating, *in vitro*, a double-stranded replicative form of the co-suppression construct from the ligated admixture of (ii), prior to introducing the resulting double-stranded replicative form into said suitable cell.

In one preferred embodiment, the vector of step (i) is an expression vector.

A library may be made in vectors that already contain eukaryotic promoters and other regulatory sequences, or a library may be made first in a prokaryotic vector and then the inverted repeats may be re-cloned into an expression vector.

The co-suppression library, therefore, comprises co-suppression constructs, comprising therein eukaryotic DNA in double-stranded form. The library may be in, for example, a prokaryotic microorganism or it may be in an isolated purified form.

When in the latter form, the co-suppression library may then be introduced into a suitable

eukaryotic cell or, more generally, a culture of eukaryotic cells or a eukaryotic cell line. The selection of eukaryotic cell is dependent on the trait for which PTGS or TGS is sought. Such traits include loss of enzyme function, alteration in cell surface receptors, change in the colour of a plant, flower or petal, an alteration in the level of resistance to a pathogen, inhibition or promotion of apoptosis, amongst many others.

The present invention contemplates, therefore, a method for identifying a eukaryotic-derived nucleic acid molecule capable of inducing PTGS or TGS in a eukaryotic cell, said method comprising:-

(i) generating a vector wherein, *in vitro*, the vector comprises a co-suppression vector comprising a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion comprising at least one unique restriction endonuclease site;

(ii) digesting the double-stranded portion of the co-suppression vector with the at least one restriction endonuclease enzyme and admixing eukaryotic-derived DNA having compatible 5' and 3' ends for ligation into the 5' and 3' ends of the digested co-suppression vector;

(iii) introducing the resulting ligated single-stranded co-suppression construct into a suitable cell to generate a double-stranded form of the co-suppression construct comprising the eukaryotic DNA; and

(iv) isolating the double-stranded co-suppression construct from said suitable cell and introducing same into a eukaryotic cell or eukaryotic cell line and screening for a trait change in said eukaryotic cell wherein the presence of a trait change is indicative of TGS or PTGS.

Generally, but not necessarily, the suitable cell in this embodiment is a prokaryotic microorganism.

With respect to each of the aforementioned embodiments and not intending to limit the present invention in any way, it is proposed that, *in vivo*, the vector of step (i) may alternatively be in the form of a single-stranded circular molecule.

Introduction of the double-stranded form of the co-suppression construct, comprising the eukaryotic-derived DNA, into a eukaryotic cell or eukaryotic cell line and permitting expression thereof *via* a promoter operable in the eukaryotic cell and operably linked upstream of the IRs results in an RNA transcript comprising double-stranded RNA with or without a stem loop. It is this which is referred to as a co-suppression effector and which is proposed to induce TGS and/or PTGS *via*, for example, RNAi.

The present invention further contemplates using a co-suppression construct, identified by the above method, for the production of transformed eukaryotic cells, tissues or group of tissues that may subsequently be regenerated into an organism exhibiting a desired trait change. Accordingly, having screened for a desired trait change, brought about by the action of a co-suppression effector in a cell following introduction into the cell of a double-stranded co-suppression construct, the double-stranded co-suppression construct that caused the desired trait change may be identified and isolated. This double-stranded co-suppression construct may then be employed in the production of stably transformed eukaryotic organisms exhibiting the desired selected trait.

The co-suppression construct or a co-suppression library may also be packaged for sale with instructions for use and/or may be provided in the form of a kit.

Kits made in accordance with the present invention may be used for the production of one or more desired IR DNA constructs, referred to herein as co-suppression constructs.

The present invention further provides cultures of prokaryotic microorganisms comprising the co-suppression constructs or co-suppression library. The present invention further provides eukaryotic cells exhibiting TGS or PTGS of a particular gene.

A further feature of the invention is the use of the co-suppression constructs and/or effectors made in accordance with the method of the invention as actives in a pharmaceutical composition. An isolated co-suppression construct and/or effector of the invention may be used as an active in a pharmaceutical composition. The nucleic acid construct and/or effector may be either DNA or RNA. Preferably, the nucleic acid is RNA. Alternatively, or in addition, an expression vector comprising a nucleic acid co-suppression construct which, when expressed, forms a co-suppression effector may also be used in a pharmaceutical composition.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of superfactants. The preventions of the action of microorganisms can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the active ingredient and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying

technique which yield a powder of active ingredient plus any additionally desired ingredient.

When the active ingredient is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet or administered *via* breast milk. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, b varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 200 mg of active compound. Alternative dosage amounts include from about 1 μg to about 1000 mg and from about 10 μg to about 500 mg. These dosages may be per individual or per kg body weight. Administration may be per hour, day, week, month or year..

The tablets, troches, pills, capsules, creams and the like may also contain the components as listed hereafter. A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of

course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and except insofar as any conventional media or agent is incompatible with the active ingredient, their use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is particularly advantageous to incorporate the active ingredient as a cream capable of preventing or delaying aging.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

General Materials and Methods

Molecular biological methods and reagents used herein which are common in the art are described in Sambrook *et al.* (Molecular Cloning. A Laboratory Manual (Cold Spring Harbor Press, 1989) and Ausubel *et al.* (Current Protocols in Molecular Biology. Eds, John Wiley & Sons, Inc., 1995-1999) both incorporated herein by reference.

EXAMPLE 2

Preparation of competent cells

Inoculate 1 L of L-broth with 1/100 volume of fresh overnight culture. Grow cells at 37°C with vigorous shaking to an A_{600} of 0.5 to 0.8. To harvest, chill the flask on ice for 15 to 30 minutes, and centrifuge at 4000 g for 15 minutes. Cells are kept as close to 0°C as possible throughout their preparation. Remove as much of the supernatant (medium) as possible. Resuspend pellets in a total of 1 L of ice-cold water. Centrifuge at 4000 g for 15 minutes. Resuspend in ~20 ml of ice cold 10% v/ glycerol. Centrifuge at 4000g for 15 minutes and resuspend to a final volume of 2 to 3 ml in ice cold 10% glycerol. This suspension may be frozen in aliquots on dry ice, and stored at -70°C.

Gently thaw the cells at room temperature and then immediately place them on ice. Remove sterile cuvettes from their pouches and place them on ice. Place the white chamber slide on ice. In a cold, 1.5 mL polypropylene tube, mix 40 μL of the cell suspension with 1 to 2 μL of DNA. Mix well and let sit on ice ~0.5 to 1 minute. Set the Gene Pulser apparatus at 25 μF. Set the Pulse Controller at 200 Ω. Set the Gene Pulser apparatus to 2.50 kV when using 0.2 cm cuvettes. Set it to 1.50 to 1.80 kV when using the 0.1 cm cuvettes. Transfer the mixture of cells and DNA to a cold electroporation cuvette, and shake the suspension to the bottom. Place the cuvette in a chilled safety chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once at the above settings.

EXAMPLE 3

Construction of a replicative DNA cloning vector

Standard molecular biological methods and reagents used for cloning are described in Sambrook *et al.* (1989, *supra*) and Ausubel *et al.* (1994, *supra*) incorporated herein by reference.

Essentially any vector, capable of being converted to a single-stranded form, can be used as a starting point into which a nucleic acid fragment may be cloned in such a manner as to generate an inverted repeat (IR) of the cloned nucleic acid. For example, a spacer DNA encoding, for example, an intron can be cloned into the multiple cloning site (MCS) of pBluescript (Stratagene, USA). A fragment of the MCS can then be cloned into this plasmid using standard molecular biological methods so that an IR of a region of the MCS flanks the spacer. pBluescript *per se* provides useful elements: an fl origin of replication and selectable marker, for example. Details on how to create such a DNA cloning vector are provided below.

The MCS from pCMV-PCR (Stratagene, USA) is another useful sequence for providing several restriction enzyme recognition sites for constructing an IR sequence. Additional useful elements, other than those present in pBluescript, can be included as part of a DNA cloning vector; for example, a spacer nucleic acid, a selectable marker and a eukaryotic and/or prokaryotic promoter, operably linked upstream of an IR. As already mentioned, a spacer nucleic acid may encode an intron. Preferably, the intron is selected to correspond to an organism which is ultimately to express a co-suppression construct as described herein. The spacer may comprise any number of nucleic acids as selected by a skilled person in the art.

One useful spacer nucleic acid comprises a polyphenol oxidase (ppo) intron. The ppo intron was amplified *via* PCR using a genomic PPO clone as template and primers ppoAcc and ppoBsi, described below. The primers contain regions of sequence complementarity to the PPO gene and have additional sequences which encode a restriction enzyme

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recognition site for *Acc*65I (GGTACC) [SEQ ID NO:1] or *Bsi*WI (CGTACG) [SEQ ID NO:2]. PCR products were digested with *Acc*65I and *Bsi*WI restriction enzymes, separated by gel electrophoresis on an agarose gel and a 640 bp fragment was purified by standard molecular biological techniques. The resulting four-base 5'-overhangs produced by both enzymes are compatible with *Acc*65I-derived overhangs.

PpoAcc	5'-GTCAGGTACCGACAGGTAATCGCGT-3'	[SEQ ID NO:3]
PpoBsi	5'-GTCTTCGTACGGGATATCACCTGTCAAAATC-3'	[SEQ ID NO:4]

pBluescript SK$^-$ DNA was digested with *Acc*65I and the 5'-termini were dephosphorylated using shrimp alkaline phosphatase. The dephosphorylated pBluescript SK$^-$ DNA was ligated with the 640 bp *Acc*65I/*Bsi*WI fragment of ppo gene and used to transform *E. coli*. Recombinants were selected, plasmid DNA was isolated, digested with *Bss*HII/*Acc*65I and the 780 bp *Bss*HII/*Acc*65I fragment (A) was purified.

pBluescript KS$^-$ was digested with *Bss*HII and *Acc*65I and the respective 140 bp *Bss*HII/*Acc*65I (B) and 2.8 kb *Bss*HII fragments were isolated. The 2.8 kb fragment was dephosphorylated (C) using shrimp alkaline phosphatase. Equimolar amounts of (A), (B) and (C) were ligated and used to transform *E. coli*. A recombinant comprising a desired DNA cloning vector, designated pIR, was selected.

Details in relation to pCMV-PCR, pBluescript and other useful vectors are commercially available and information in relation to these vectors may be provided by the supplying company. Such commercial product information is herein incorporated by reference.

DNA cloning vectors containing IRs and useful sites can be created in other ways. The following describes another method of creating such a vector, starting with pCMV-PCR.

pCMV-PCR plasmid DNA was digested with *MluI*/*Dra*III and the 5'-termini were dephosphorylated by treatment with shrimp alkaline phosphatase. The multiple cloning site of pCMV-PCR was amplified *via* PCR, using the oligonucleotide primers MCS-Sac-Dra

and MCS-Kpn-MluI. The amplified MCS fragment was purified with a QIAquick PCR purification column and digested with *Dra*III and *Mlu*I. The 150 bp *Dra*III/*Mlu*I MCS fragments were gel purified and ligated to the *Mlu*I/*Dra*III-digested pCMV-PCR. The ligation mix is used to transform *E. coli* cells. Recombinant clones, which contain an inverted repeat of the multiple cloning site, separated by a spacer region of 440 bp, are selected. The resultant vector is designated pCMV-IR. The *Bbv*CI site within pCMV-IR (generated from the MCS-Sac-Dra PCR primer) allows production of single-stranded IR cloning vector DNA *via* nicking with N.BbvCIA or N.BbvCIB, followed by Exonuclease III digestion, similar to the process described in Example 6.

Oligonucleotide sequences:

MCS-Sac-Dra 5'-GGGAACACGTAGTGCTGAGGAGCTCCACCGCGGTGGC-3' [SEQ ID NO:7]

MCS-Kpn-Mlu 5'-ACTTAACGCGTACCGGGCCCCCCCTC-3' [SEQ ID NO:8]

The inverted repeats within the final vector contain two *Sap*I sites. Digestion of annealed single-stranded form of pCMV-IR with *Sap*I generates a vector stem/loop, a smaller spacer stem loop and a very small linear double-stranded fragment. The *Sap*I-digested vector stem loop terminus contains a 5'-CTT-overhang, the spacer/loop terminus contains a 5'-CGG-overhang and the released double-stranded linear fragment contains 5'-AAG- and 5'-CCG-overhangs. The linear fragment can conveniently be removed from the restriction digestion by, for example QIAquick PCR column purification.

Ligation of added double-stranded DNA (eg. cDNA or genomic DNA) with compatible ends (i.e.: containing a 5'-AAG- and a 5'-CCG-overhang) to the pCMV-IR stem/loop fragments, followed by conversion to double-stranded form results in the creation of inverted repeat constructs of the added DNA fragments. The vector stem/loops cannot ligate to each other and the spacer stem/loops cannot ligate to each other, and the added DNA fragments cannot ligate to each other. Double-stranded DNA fragments with compatible ends for cloning into pCMV-IR can be made by addition primer sequences *via*

PCR, ligation of adaptor sequences or by first cloning blunt-end fragments into *Eco*RV-cut pCMV-PCR, followed by digestion with *Sap*I and purification of resultant fragments.

Further modifications to the pCMV-IR vector can be made to reduce background resulting from undigested pCMV-IR in the ligations of *Sap*I-digested single-stranded vector to compatible double-stranded DNA fragments. Cloning of a 'killer' gene such as *ccdB* between the *Sap*I sites of the inverted repeats creates an inverted repeat of the *ccdB* gene, which is removed from the vector by *Sap*I digestion. An inverted repeat cloning vector containing the *ccdB* gene can be propagated in DB3.1 *E. coli* cells and then converted into single-stranded inverted repeat cloning vector. However, any plasmids containing the *ccdB* gene are not able to propagate in wild-type *E. coli* strains which lack an F' episome such as DH5-alpha or TOP10. This provides an efficient negative selection method for eliminating non-recombinant clones from an inverted repeat cloning experiment.

A DNA cloning vector may comprise the following:

(i) Restriction endonuclease recognition sites, T-overhang, LIC, and other useful sites that may be selected by a skilled person in the art.

(ii) An IR which flanks both sides of a 'spacer' region of DNA. Preferably the spacer is longer than 300 bp. More preferably the spacer encodes an intron.

(iii) The capability for single-stranded rescue of the vector and insert, *via* filamentous bacteriophage DNA replication control elements (e.g. the intergenic region from f1 phage or a vector constructed from a filamentous phage such that rescue is not needed).

(iv) A fully functional replicon so that replication of the vector may occur in a permissive host organism.

(v) A site for a "nicking" restriction enzyme, such as N.*Bpu*10I, N.*Bbv*CIA or N.*Bbv*CIB.

An example of cloning steps to produce a DNA cloning vector is illustrated diagrammatically in Figure 7. Vectors and DNA fragments represented are double-stranded.

EXAMPLE 4

Removing or changing a Spacer region (Intron) Nucleic Acid

As described in Example 3, above, spacer nucleic acid may be added during the production of DNA cloning vector. A spacer nucleic acid may allow replication in bacteria. Intron-encoding spacers appear to provide greater PTGS efficiency. However, a spacer is not necessarily required if the IR is small (e.g. less than 75 nucleotides), as shown in Figure 5. Small inverted repeats are generally more stable in sbcC mutant strains of *E. coli*, such as Sure™ cells.

Removing from or changing a spacer in a DNA cloning vector can be readily achieved. The spacer can be replaced by digestion of the vector with a restriction enzyme that cleaves either side of the spacer, within the IRs. A new spacer DNA fragment with compatible ends can then be added by ligation.

Removal of the spacer altogether can be achieved by self-ligation of the DNA cloning vector after the spacer is removed by restriction digestion.

A spacer can also be replaced in the single-stranded co-suppression vector – see Example 5, below – by ligation of a spacer stem-loop with a compatible replicon (plasmid) stem-loop fragment.

EXAMPLE 5

Generation of a co-suppression vector via single-stranded helper phage

(a) Growth of bacteria

E. coli strain used was XL-1 Blue XRF' (Δ(mcrA)183 Δ (mcrCB-hsdSMR-mrr)173endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacI^q Z ΔM15 Tn10 (Tet^r)]). Bacterial strains were grown in under standard conditions. Helper phage VCSM13 (Stratagene) was used for rescue of single-stranded phagemids.

(b) Single-stranded phagemid rescue and purification

Inoculate a single colony into 5 mL of 2YT containing 50 mg/mL ampicillin and VCSM13 helper phage at 10^7 -10^8 pfu/mL (multiplicity of infection ~10). Grow the culture at 37°C with vigorous aeration for 16 hours, or until growth has reached saturation. If using VCSM13, after 1-2 hours, add kanamycin to 70 µg/mL to select for infected cells. Centrifuge 1.5 mL of the cell culture for 5 minutes n a microcentrifuge. Remove 1 mL of the supernatant to a fresh tube, then add 150 µL of a solution containing 20% PEG8000 and 2.5 M NaCl. Allow phage particles to precipitate on ice for 15 minutes. Centrifuge for 5 minutes in a microcentrifuge. Remove supernatant and resuspend the pellet in 400 µL of 0.3 M NaOAc (pH 6.0) and 1 mM EDTA by vortexing vigorously. Extract with 1 volume of phenol-chloroform and centrifuge for 1-2 minutes to separate phases. Transfer the aqueous phase to a fresh tube and add 1 mL of ethanol. Centrifuge for 5 minutes. Remove ethanol and dry the pellet comprising the single-stranded form of the DNA cloning vector. Dissolve the pellet in 25 µL of TE buffer.

(c) Digestion of 'single-stranded' co-suppression vector

To ensure the production of a partially double-stranded stem portion within the 'single-stranded' co-suppression vector DNA, phagemid DNA was heated to 90°C for 3 minutes and allowed to cool slowly to room temperature (30-60 minutes). An aliquot of this sample

was digested with 2 units of restriction enzyme per microgram of phagemid DNA in a volume of 20 μL for 1 hour.

EXAMPLE 6

Generation of a co-suppression vector, in vitro

To generate a co-suppression vector *in vitro*, the following components were added to the reaction tube:

plasmid containing Bpu10I site (10μg)	10-184μl
10X R+ reaction buffer	20 μl
N.Bpu10I (5 u/μl)	2 μl
Deionized water up to	200 μl

The tube was vortexed and spun in a microcentrifuge for 3-5sec and then incubated at 37°C for 1 hr. Following incubation, 1 2 volumes of phenol (100 μl) and 1 2 volume of chloroform/isoamyl alcohol (24:1) (100 μl) were added, and the mixture was, vortexed for 10 sec and then centrifuged at maximum speed for 5 min.

The upper aqueous phase was transferred to a fresh tube and 1 volume (200 μl) of chloroform/isoamyl alcohol (24:1) was added. The mixture was vortexed and centrifuged for 5 minutes. Following two further extractions with chloroform/isoamyl alcohol, the upper aqueous phase was transferred to a fresh tube. A 1/10 volume of 3M sodium acetate and 2.5 volumes of ice-cold ethanol were added and the mixture incubated at -20°C for 1 hr. Following incubation, the mixture was centrifuged at maximum speed for 10 minutes.

The supernatant was poured off and the DNA pellet carefully washed with 200 μl 75% ice-cold ethanol, dried and dissolved in 25 μl of deionized water. Exonuclease III treatment was achieved by adding to the 25 μl nicked DNA the following components: 12.5 μl 10X ExoIII reaction buffer, 3 μl Exonuclease III (200 U/μl) and 5 μl deionized water. This

mixture was incubated for 15 min at 37°C and the reaction stopped by heating stopped by heating at 70°C for 10 min.

Finally, the resulting DNA was cleaned by purification using a QIAquick PCR kit (Qiagen) and eluted in 30 µl EB or deionized water.

EXAMPLE 7

Ligation to double-stranded DNA fragments to form a co-suppression construct

Double-stranded DNA fragments with compatible ends are added to the purified restriction enzyme digested products of the 'single-stranded' co-suppression vector and ligated using standard conditions (Sambrook *et al.*, 1989, *supra)*.

Co-suppression constructs can be created by cloning double-stranded DNA fragments into the IR region of the co-suppression vector. Conversion of the single-stranded recombinant DNA co-suppression construct into the 'double-stranded' form generates an IR of the cloned DNA. The cloned DNA fragment may be inserted into the IR region or may replace part of the IR region. These DNA fragments may be cloned in a variety of ways, some of which are outlined below and illustrated in Figures 2-4.

(a) Single restriction enzyme cutter

A co-suppression vector is digested with a restriction enzyme that cleaves within the double-stranded IR region to produce plasmid (replicon) and spacer loops. DNA fragments comprising compatible ends are ligated to the loops to generate a new double-stranded IR region.

One method to increase efficiency of recombinants is to use a DNA cloning vector which comprises a restriction enzyme recognition site which occurs infrequently in genomic sequences (e.g. *Srf*I). Blunt-ended DNA fragments can then be ligated with *Srf*I-digested DNA cloning vector in the presence of *Srf*I enzyme. *Srf*I sites regenerated by ligation of

the stem-and-loops to each other are cut by *Srf*I, whereas recombinants arising from the ligation of double-stranded DNA fragments to the stem-and-loops destroy the *Srf*I site. *Srf*I-containing fragments are not clonable using this method.

(b) Directional cloning with two restriction enzymes

A co-suppression vector is digested with two restriction enzymes that cleave the double-stranded IR region to produce a plasmid (replicon) stem-loop portion and a spacer stem-loop portion and double-stranded DNA fragment(s) arising from the cleavage. DNA fragments containing compatible ends are ligated to the loops to generate a new double-stranded region. Using two different restriction enzyme sites overcomes the problem of the unwanted re-ligation of the plasmid and spacer loops to each other.

(c) TA-cloning method

A co-suppressionvector is digested with a restriction enzyme that cleaves within the IR region to produce plasmid (replicon) and spacer stem-loop portions. Single T 3'-overhangs are added by one of the following methods:

1. digestion of plasmid with e.g. *Xcm*I sites engineered to produce single T 3'-overhangs at the stem termini; or

2. incubation with DNA polymerase in the presence of dTTP (Marchuk *et al., Nucleic Acids Research 10:* 1154, 1991). The plasmid DNA molecules are then ligated with DNA fragments containing single A 3'-overhangs. A-overhangs can be conveniently generated during or after PCR amplification of DNA fragments.

(d) LIC adaptors

A co-suppression vector is digested with a restriction enzyme (e.g. *Sap*I) that cleaves within the IR region to produce plasmid and spacer stem-loop portions. The digested DNA

is treated with a DNA polymerase in the presence of limiting dNTPs (e.g. only dTTP) to generate single-stranded overhangs. Double-stranded DNA containing single-stranded overhangs compatible with the prepared vector stem-loop portions is annealed with the vector portions to form the co-suppression construct.

Co-suppression constructs generated by any of these means may be used to transform bacteria such as, for example *E. coli*. Therein the constructs are converted to the double-stranded form, producing a library of co-suppression constructs (ii) (see Example 8, below).

EXAMPLE 8

Generation of stem and loop structures by PCR

Stem-and-loop structures can also be generated *via* PCR amplification (see Figure 6). As an example, the following oligonucleotides were synthesized to amplify a pineapple polyphenol oxidasae (PPO) intron:

PPO-Srf-F:	5'-CCCGTGCTCCGACAGgtaatcgcgttag-3'	[SEQ ID NO:5]
PPO-Srf-R:	5'-CCCGTGCTCCATCACctgtcagggtcgcaat-3'	[SEQ ID NO:6]

The underlined sequences are not present in the PPO gene sequence but were added to create terminal IRs in the PCR amplification products.

The pineapple PPO intron as amplified *via* PCR using the oligonucleotides PPO-Srf-F and PPO-Srf-R as primers. Further amplification in the presence of only PPO-Srf-F results in the amplification of only one DNA strand, leading to the production of predominantly single-stranded DNA flanked by 10-nucleotide IRs. Incorporation of the underlined sequence into the amplification products can form stem and loop structures. These can be ligated to blunt-ended double-stranded DNA fragments and/or to replicon-stem portions to form a co-suppression construct.

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In an alternative approach, a single-stranded spacer-loop with self-complementary ends (i.e. the double-stranded stem portion) may be ligated to a single-stranded cDNA polynucleotide and used to prime second strand synthesis. A similar approach may be taken using single-stranded genomic DNA or PCR fragments. Once the double-stranded stem portion has been generated, these may again be ligated to double-stranded DNA fragments and/or to replicon-stem portions to form a co-suppression construct.

EXAMPLE 9

Hybrid co-suppression vectors

Hybrid co-suppression vectors are generated comprising replicons (plasmids) and stem-loops from different co-suppression vectors. In one embodiment, a co-suppression vector is subjected to rolling-circle replication *in vitro* to generate a concatamer comprising multiple repeats of a strand of the co-suppression vector. The concatamer is subjected to annealing conditions to provide a nucleic acid complex having multiple stem-loops portions. This complex is used as a source of stem-loops for ligation to a suitably-digested co-suppression vector.

In an alternative embodiment, the starting material comprises single dsDNA cloning vectors. Stem-loops may also be generated *in vitro via*, for example, the use of PCR. In a preferred embodiment, replicon stem-loops and spacer stem-loops each contain a different selectable marker. For example, ligation of a replicon stem-loop containing an ampicillin resistance gene and a spacer stem-loop containing a kanamycin resistance gene generates a recombinant vector which is both ampicillin resistant and kanamycin resistant. When the two stem-loops are generated, derived from separate plasmids, and ligated in the presence of ds fragments, recombinants may easily be selected, eliminating the background of non-recombinants.

EXAMPLE 10

Conversion of recombinant co-suppression construct (i) to double-stranded form (ii)

The ligation mix comprising co-suppression constructs (i) is used to transform *E. coli*. The recombinant co-suppression construct replicates in *E. coli* and is converted to the double-stranded form (ii) and, in the process, generates an IR of the cloned DNA fragment flanking the spacer region (refer to Figure 4).

A single stranded co-suppression construct (i) can also be converted to a predominantly double-stranded form (ii) *in vitro*, prior to bacterial transformation. Conversion to double-stranded form can be achieved by annealing a complementary oligonucleotide primer and extending with a DNA polymerase such as Taq.

A PCR reaction mix consisted of:

10 µl of annealed single-stranded co-suppression vector,
5 µl of 10x PCR reaction buffer,
1 U Taq DNA polymerase,
0.2 µM primer,
250 µM each dATP, dCTP, dGTP, dTTP,
deionized water to make up final reaction volume to 50 µl.

The reactions were cycled as follows: 95°C for 30 sec, 50°C for 30 sec, 72°C for 7 min. Reactions were cleaned up using QIAgen mini-elute columns: purified DNA was eluted in 10 µl EB buffer and used for *E. coli* transformation. Similar PCR conditions were also used to convert single-stranded ligated DNA to double-stranded form before transformation of *E. coli*.

EXAMPLE 11

Creation of a co-suppression library via shot-gun cloning

A library of DNA fragments (cDNA or genomic) corresponding to a single gene or genetic cluster, may be generated by isolating the DNA, restricting the DNA to generate a range of differently-sized fragments, size-fractionating the fragments and selecting a particular size range thereof for cloning into a suitably-digested co-suppression vector. The double-stranded DNA fragments can be generated in a variety of ways familiar to those skilled in the art. These methods include restriction digestion, sonication, partial cleavage with DNAse I, PCR amplification of template DNA, synthesis of cDNA from RNA. Double-stranded DNA fragments with termini compatible with cloning into a co-suppression vector can be generated in many ways, including restriction digestion, adaptor ligation, PCR amplification and end-repair of DNA fragments.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

BIBLIOGRAPHY

Ausubel *et al., Current Protocols in Molecular Biology*, Eds. John Wiley & Sons, Inc., 1995-1999

Bingham, *Cell 90:* 385-387, 1997;

Caplen *et al., Gene 252:* 95-105, 2000;

Cogoni and Mancino, *Proc. Natl. Acad. Sci. USA 94:* 10233-10238, 1997;

Cogoni *et al., EMBO J. 15:* 3153-3163, 1996;

Elbashir *et al., Nature 411:* 494-498, 2001;

Fire *et al., Nature 391:* 806-811, 1998;

Forthler and Belote, *Genesis 264:* 240-244, 2000;

Lindbo *et al., The Plant Cell 5:* 1749-1759, 1993;

Marchuk *et al., Nucleic Acids Research 10:* 1154, 1991;

Napoli *et al., The Plant Cell 4:* 279-289, 1990;

Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989;

Schoppmeier and Daman, *Development Genes and Evolution 211:* 76-82, 1001;

Singh *et al., Biochemical Society Transactions 28:* 925-927, 2000;

Smith *et al., Nature 407:* 319-320, 2000;

Timmons and Fire, *Nature 395:*854, 1998;

Timmons *et al., Gene 263:* 103-112, 2001;

van der Krol *et al., The Plant Cell 4:* 291-299, 1990;

Waterhouse *et al., Proc. Natl. Acad. Sci. USA 95:* 13959-13964, 1999;

CLAIMS

1. A method for generating a library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) converting a double-stranded replicative circular DNA cloning vector, comprising an inverted repeat (IR) sequence, into a single-stranded form;

(ii) treating said single-stranded form such that self-complementary sequences derived from said IR sequence anneal to form a region of double-stranded nucleic acid;

(iii) cleaving said double-stranded region formed in step (ii) by one or more restriction enzymes to form a vector stem-loop portion and a spacer stem-loop portion;

(iv) ligating said stem-loop portions of step (iii) with double-stranded DNA fragments containing termini compatible with said vector and spacer stem-loops to form recombinant nucleic acid molecules; and

(v) converting recombinant nucleic acid molecules of step (iv) into a double-stranded circular form.

2. A method of Claim 1, wherein the double-stranded region formed in step (ii) contains at least one restriction enzyme recognition site.

3. A method of Claim 1, wherein said nucleic acid molecules of step (iv) are converted into a double-stranded circular form *in vitro*.

4. A method of Claim 1, wherein said nucleic acid molecules of step (iv) are converted into a double-stranded circular form by transformation of a host cell capable of carrying out said conversion as part of the replicative process.

5. A method of any one of Claims 1 to 4, wherein said suitable cell is a prokaryotic microorganism.

6. The method of any one of Claims 1 to 4, wherein said suitable cell is a eukaryotic cell.

7. A method for generating a library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a single-stranded replicon portion and a single-stranded loop portion separated by double-stranded stem portion comprising at least one restriction endonuclease site;

(ii) digesting said partially single-stranded vector with at least one restriction endonuclease and admixing therewith double-stranded genomic DNA or cDNA derived from a eukaryotic cell and digested with the same restriction endonuclease or other enzyme or under conditions providing compatible 3' and 5' end portions for ligation into the restricted partially single-stranded vector and subjecting said admixture to ligation conditions to generate the partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) introducing the ligated admixture of (ii) into said suitable cell under conditions to permit the generation of a double-stranded replicative

form of said partially single-stranded vector comprising double-stranded genomic DNA or cDNA fragments.

8. The method of Claim 7, wherein the double-stranded replicative form of step (iii) is first generated *in vitro* from the ligated admixture prior to introducing said double-stranded form into said cell.

9. The method of Claim 7 or Claim 8, wherein said suitable cell is a prokaryotic microorganism.

10. The method of Claim 7 or Claim 8, wherein said suitable cell is a eukaryotic cell.

11. The method of Claim 7 or Claim 8, wherein said double-stranded stem portion arises from self-annealing of complementary sequences derived from inverted repeat sequences in a DNA cloning vector.

12. The method of Claim 7 or Claim 8, wherein the generation of the vector of step (i) is initiated by first obtaining a double-stranded DNA cloning vector having a multiple cloning site such that, upon digestion, said cloning vector is cleaved only within the multiple cloning site.

13. The method of Claim 12, wherein a spacer nucleic acid molecule is cloned within the multiple cloning site of the DNA cloning vector.

14. The method of Claim 13, wherein the spacer comprises an intron.

15. The method of any one of Claims 12, 13 or 14, wherein the DNA cloning vector comprises restriction sites adjacent both sides of said spacer.

16. The method of Claim 15, wherein the restriction sites facilitate directional cloning of digested DNA fragments.

17. The method of any one of Claims 12 to 15, wherein said DNA cloning vector comprises two homologous nucleotide sequences flanking said spacer such that, when in single-stranded form, said spacer permits said homologous sequences to anneal together to create a partially double-stranded molecule.

18. The method of any one of Claims 12 to 16, wherein said DNA cloning vector is capable of generating single-stranded replicative intermediates in the presence of a helper phage.

19. The method of Claim 18, wherein said replicative intermediate is generated by nicking one strand of said double-stranded DNA cloning vector and digesting said nicked strand with an exonuclease.

20. The method of any one of Claims 7 to 19, wherein said suitable cell supports formation of a single-stranded replicative form *via* the use of a helper phage.

21. The method of Claim 20, wherein said suitable cell is a prokaryotic microorganism.

22. The method of Claim 7 or Claim 8, wherein, *in vivo*, the vector of step (i) is in the form of a single-stranded circular molecule.

23. A co-suppression construct comprising two single-stranded DNA loop portions separated by a double-stranded portion wherein the double-stranded portion comprises one or more restriction endonuclease sites into which has been introduced a double-stranded DNA fragment derived from a eukaryotic cell.

24. The co-suppression construct of Claim 23, wherein the eukaryotic cell is derived from a species from the list consisting of plants, invertebrate animals such as insects and nematodes, and vertebrate animals such as mice, livestock and humans.

25. The co-suppression construct of Claim 23 or Claim 24, wherein said construct is converted into a double-stranded form in a suitable cell.

26. The co-suppression construct of Claim 25, wherein said suitable cell is a prokaryotic microorganism.

27. The co-suppression construct of Claim 25, wherein said suitable cell is a eukaryotic cell.

28. A method for generating a co-suppression library of viral- or eukaryotic-derived nucleic acid molecules in a suitable cell, said method comprising the steps of:-

(i) generating a vector wherein, *in vitro*, the vector comprises a co-suppression vector having a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion comprising at least one restriction endonuclease site;

(ii) digesting the double-stranded portion of the vector with the at least one restriction endonuclease and admixing therewith a double-stranded genomic DNA or cDNA preparation digested with the same restriction endonuclease or with an enzyme or under conditions providing compatible 3' and 5' end portions for ligation into the restricted co-suppression vector and subjecting said admixture to ligation conditions to generate the co-suppression construct comprising double-stranded genomic DNA or cDNA fragments inserted into the double-stranded portion of said vector; and

(iii) introducing the ligated admixture of (ii) into a suitable cell under conditions to permit the generation of a double-stranded replicative form of said co-suppression construct.

29. The method of Claim 28, wherein the double-stranded replicative form of step (iii) is first generated *in vitro* from the ligated admixture prior to introducing said double-stranded form into said suitable cell.

30. The method of Claim 28 or Claim 29, wherein said suitable cell is a prokaryotic microorganism.

31. A co-suppression library comprising co-suppression constructs of Claim 28 or 29, wherein said co-suppression constructs comprise therein eukaryotic-derived DNA in double-stranded form.

32. The co-suppression library of Claim 31 in a prokaryotic microorganism.

33. The co-suppression library of Claim 31 in isolated purified form.

34. The isolated co-suppression library of Claim 33, comprised in eukaryotic cells or a culture of cells or a cell line.

35. A nucleic acid molecule isolated from the co-suppression library of Claim 33 or Claim 34.

36. A method for identifying a eukaryotic-derived nucleic acid molecule capable of inducing PTGS or TGS in a eukaryotic cell, said method comprising:-

(i) generating a vector wherein, *in vitro*, the vector comprises a co-suppression vector comprising a single-stranded loop portion and a single-stranded replicon portion separated by a double-stranded portion

comprising at least one unique restriction endonuclease site;

(ii) digesting the double-stranded portion of the co-suppression vector with the at least one restriction endonuclease enzyme and admixing eukaryotic-derived DNA having compatible 5' and 3' ends for ligation into the 5' and 3' ends of the digested co-suppression vector;

(iii) introducing the resulting ligated single-stranded co-suppression construct into a suitable cell to generate a double-stranded form of the co-suppression construct comprising the eukaryotic DNA; and

(iv) isolating the double-stranded co-suppression construct from the suitable cell and introducing same into a eukaryotic cell or eukaryotic cell line and screening for a trait change in said eukaryotic cell wherein the presence of a trait change is indicative of TGS or PTGS.

37. A method of Claim 36, wherein the vector of step (i) is an expression vector.

38. The method of Claim 37, wherein, *in vivo*, the vector of step (i) is in the form of a single-stranded circular molecule. /

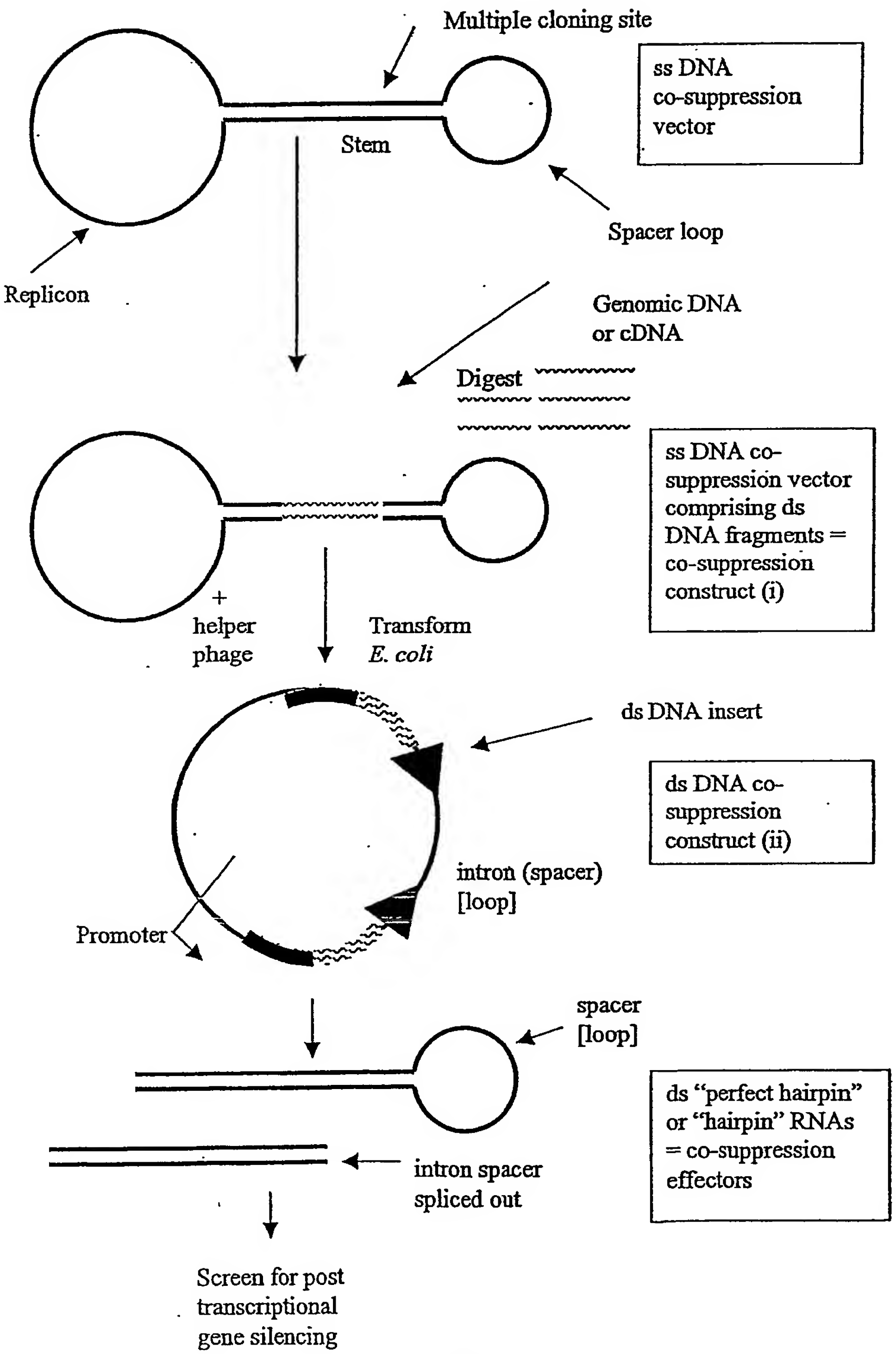
39. The method of Claim 36 or Claim 37, wherein said suitable cell is a prokaryotic microorganism.

40. The method of Claim 36 or Claim 37, wherein said suitable cell is a eukaryotic cell.

41. Use of a co-suppression construct identified by the method of Claim 36 in the production of transformed eukaryotic cells, tissues or group of tissues that may subsequently be regenerated into an organism exhibiting a desired trait change.

42. A co-suppression library in the form of a kit packaged for sale and with instructions for use.

43. The kit of Claim 42, wherein said kit is used for the production of inverted repeat DNA constructs.



Multiple cloning site
ss DNA co-suppression vector
Stem
Spacer loop
Replicon
Genomic DNA or cDNA
Digest
ss DNA co-suppression vector comprising ds DNA fragments = co-suppression construct (i)
+ helper phage
Transform E. coli
ds DNA insert
ds DNA co-suppression construct (ii)
intron (spacer) [loop]
Promoter
spacer [loop]
ds "perfect hairpin" or "hairpin" RNAs = co-suppression effectors
intron spacer spliced out
Screen for post transcriptional gene silencing

Figure 1

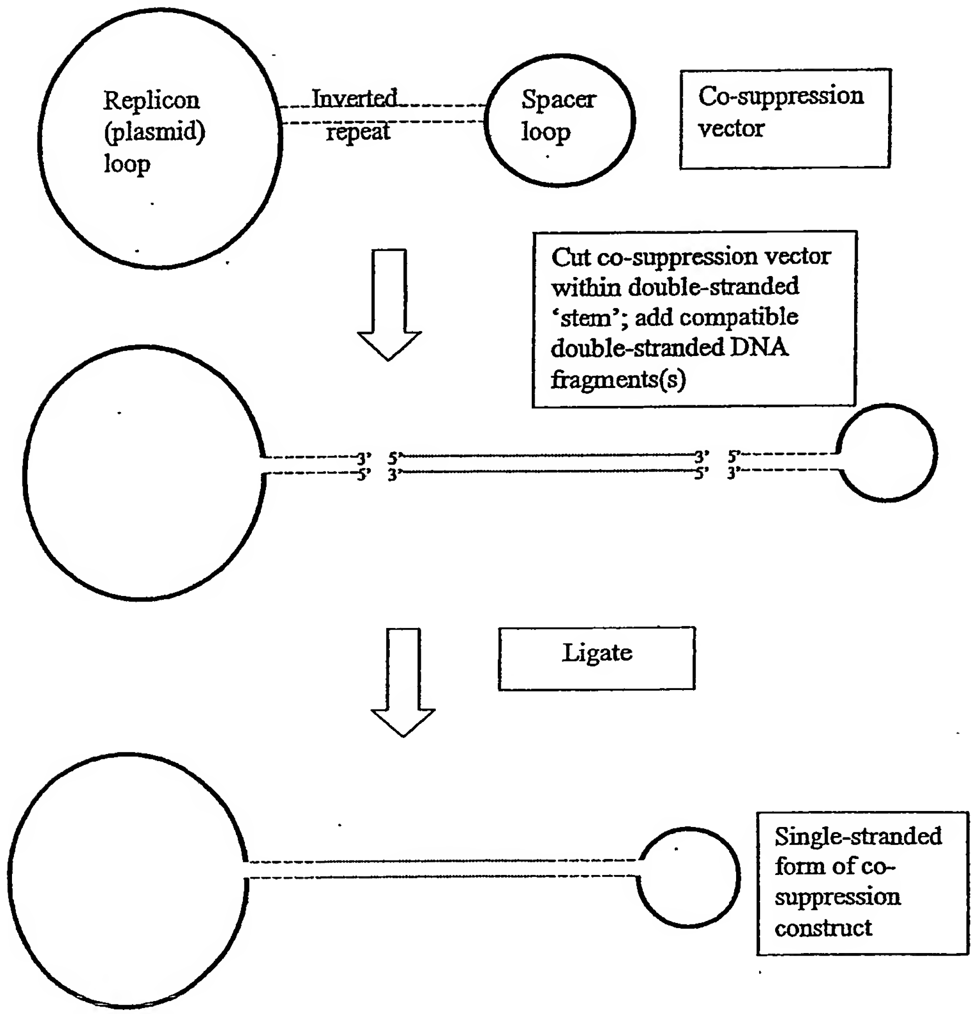

Replicon (plasmid) loop
Inverted repeat
Spacer loop
Co-suppression vector
Cut co-suppression vector within double-stranded 'stem'; add compatible double-stranded DNA fragments(s)
3'
5'
5'
3'
3'
5'
5'
3'
Ligate
Single-stranded form of co-suppression construct

Figure 2

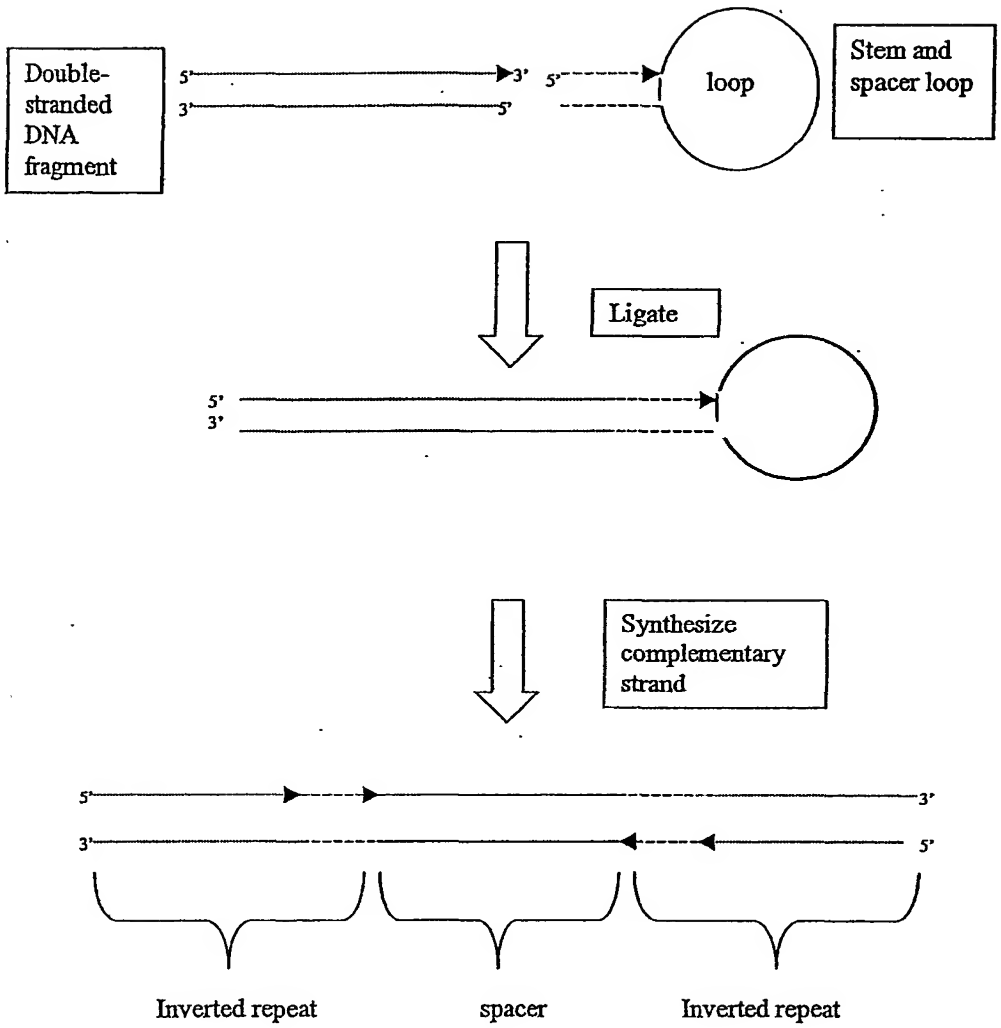


Figure 3

NUC 39187

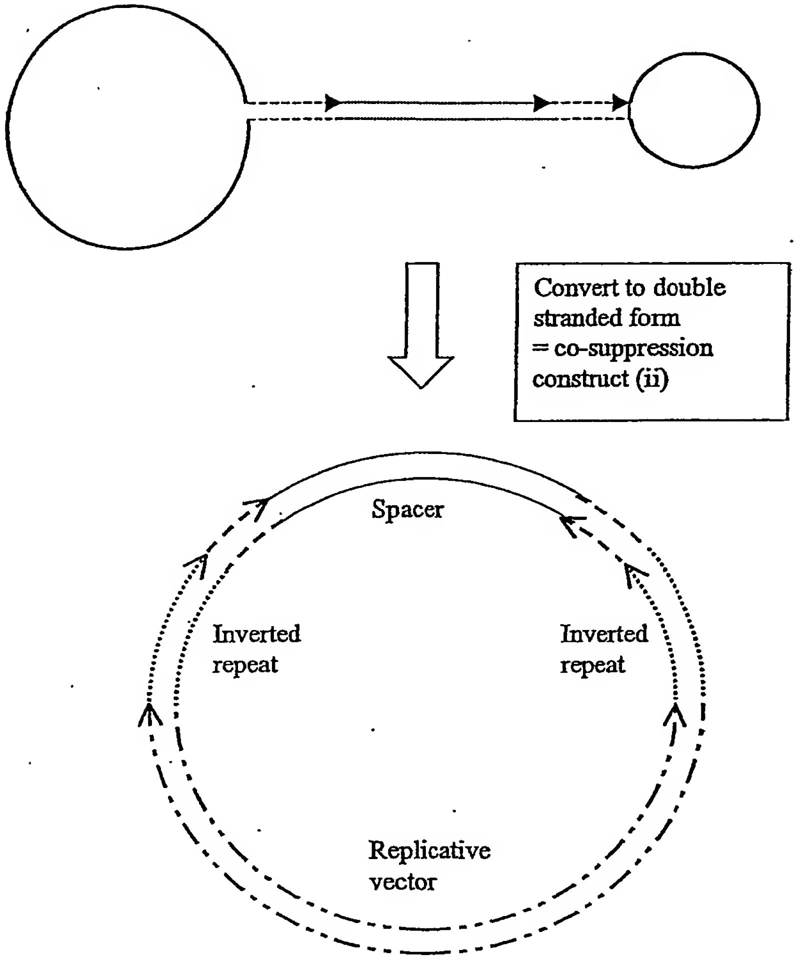

Convert to double stranded form = co-suppression construct (ii)
Spacer
Inverted repeat
Inverted repeat
Replicative vector

Figure 4

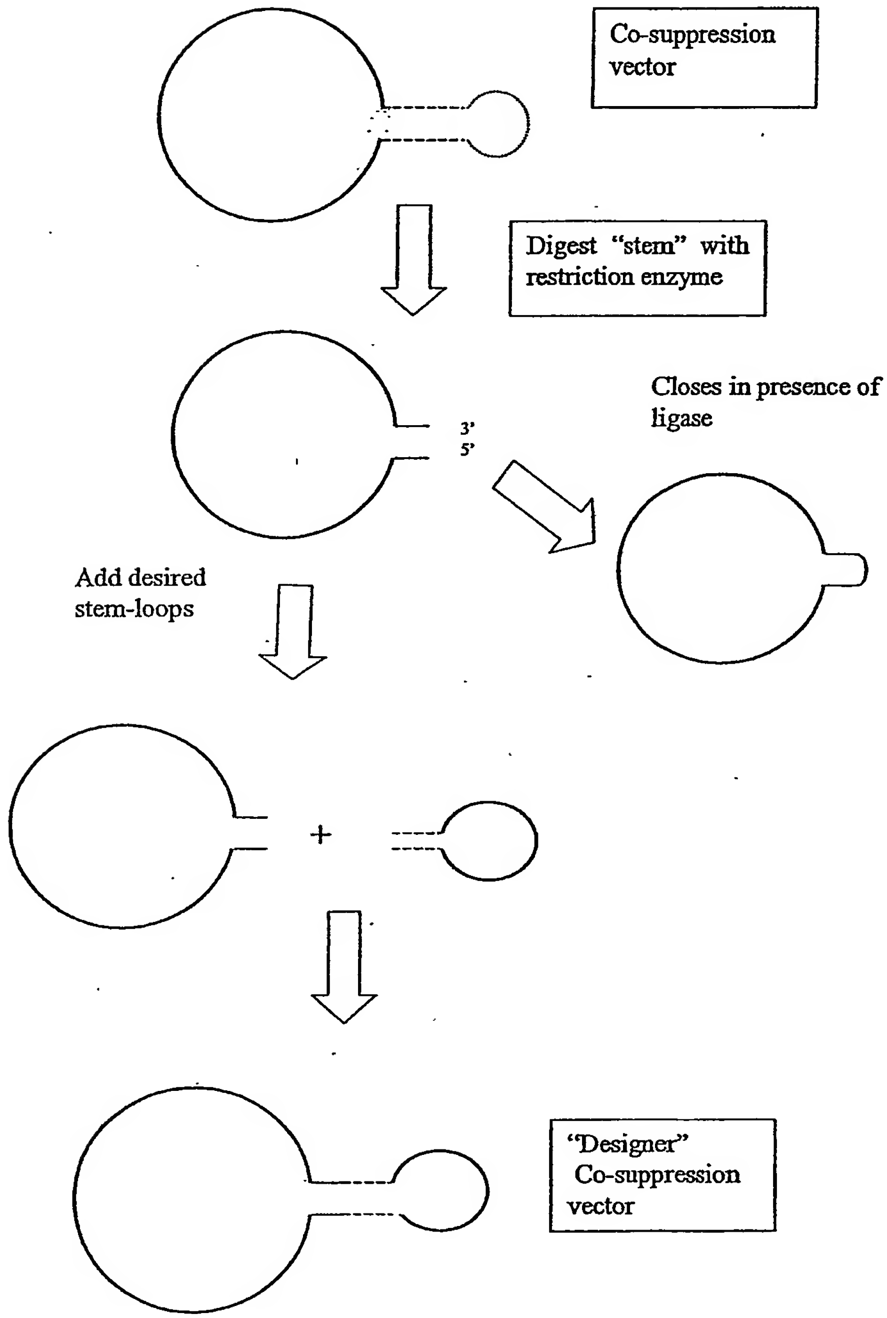


Figure 5

NUC 39189

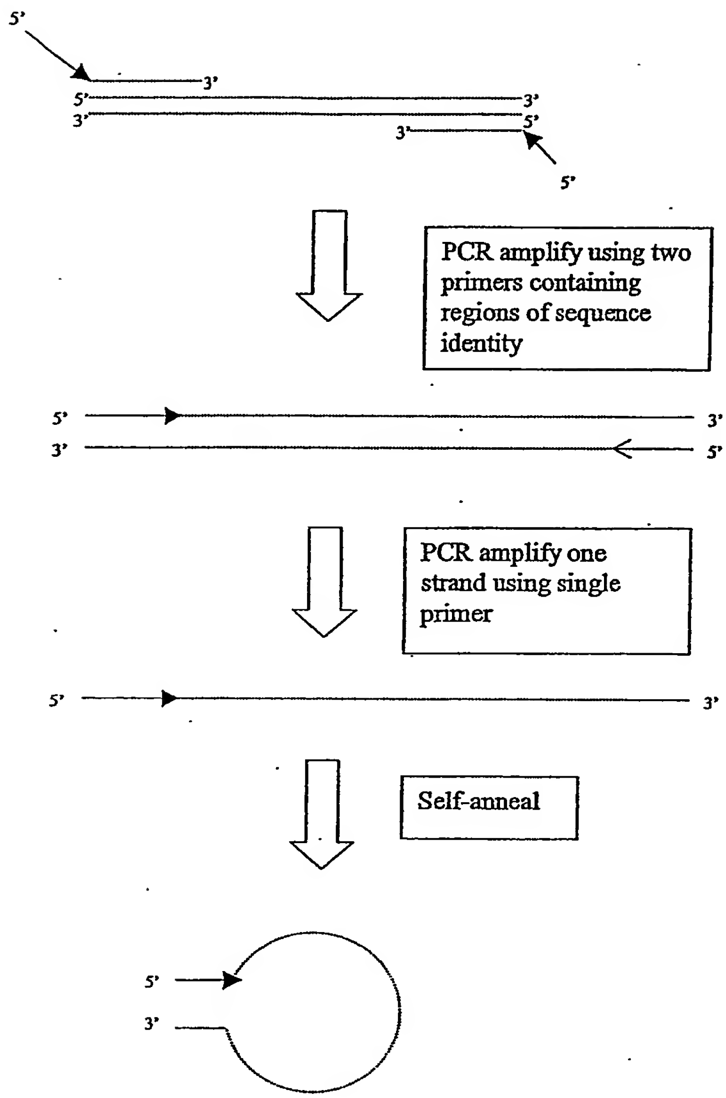

5'
3'
5'
3'
3'
5'
3'
5'
PCR amplify using two primers containing regions of sequence identity
5'
3'
3'
5'
PCR amplify one strand using single primer
5'
3'
Self-anneal
5'
3'

Figure 6

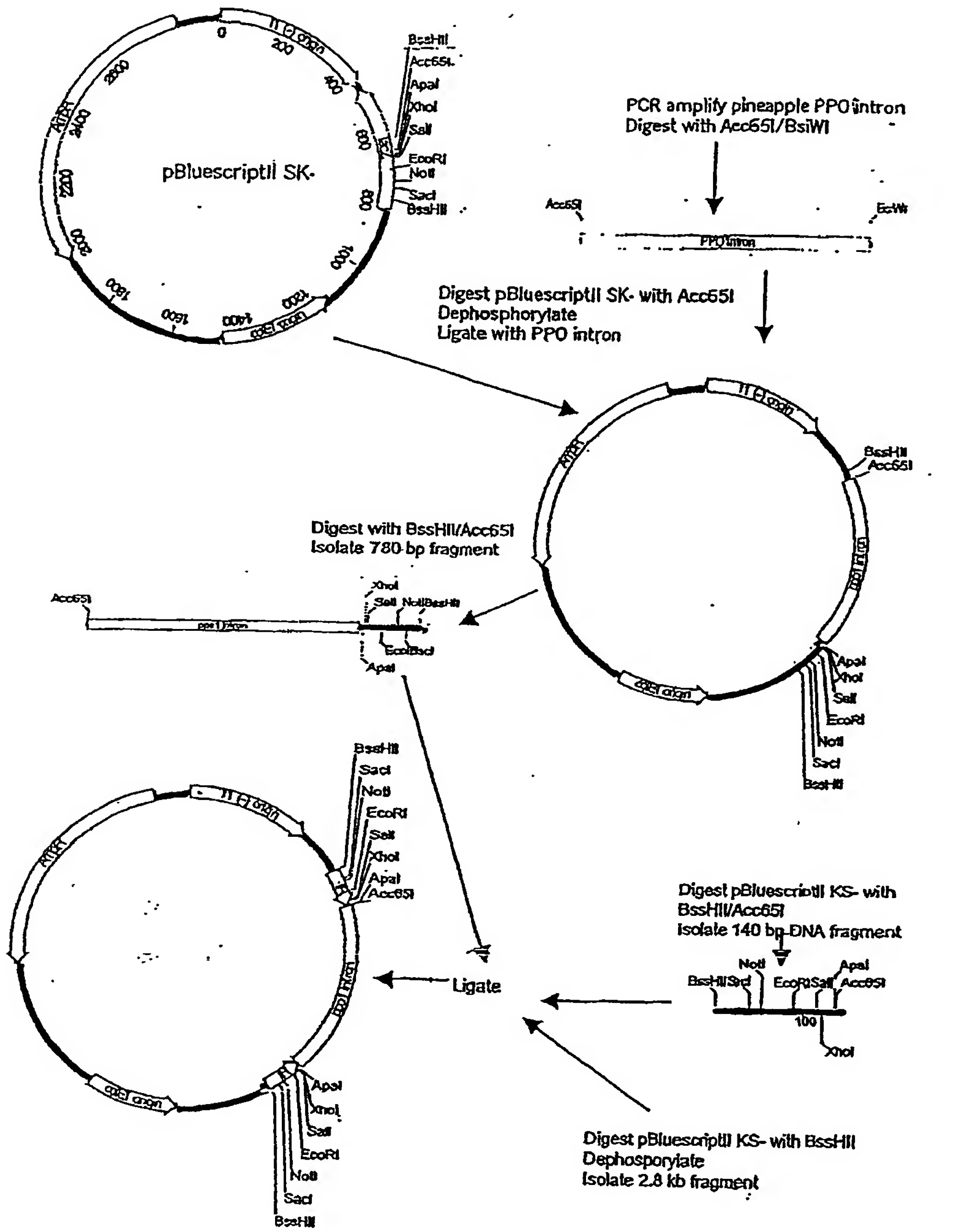


Figure 7

NUC 39191

SEQUENCE LISTING

<110> Holton, Timothy A

<120> A method for generating a nucleic acid library

<130> 2574651/EJH

<140> PCT/AU02/01326
<141> 2002-09-27

<150> AU PR7966
<151> 2001-09-27

<150> AU 20002951039
<151> 2002-08-28

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<170> PatentIn version 3.1

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NUC 39192

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 2574651/EJH/sjw	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/AU02/01326	International filing date *(day/month/year)* 27 September 2002	(Earliest) Priority Date *(day/month/year)* 27 September 2001
Applicant HOLTON, Timothy Albert		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:
      [ ] contained in the international application in written form.
      [ ] filed together with the international application in computer readable form.
      [ ] furnished subsequently to this Authority in written form.
      [ ] furnished subsequently to this Authority in computer readable form.
      [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished
2. [X] Certain claims were found unsearchable (See Box I).
3. [ ] Unity of invention is lacking (See Box II).
4. With regard to the title, [ ] the text is approved as submitted by the applicant.
   [X] the text has been established by this Authority to read as follows:

   STEM-LOOP VECTOR SYSTEM
5. With regard to the abstract, [ ] the text is approved as submitted by the applicant
   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is Figure No.
   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure
   [ ] because this figure better characterizes the invention
   [ ] None of the figures

NUC 39194

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01326

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int. Cl. [7]:	C12N 15/66
According to International Patent Classification (IPC) or to both national classification and IPC	
B.	**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC (WPIDS), CA

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, MEDLINE, CA, BIOSIS: Keywords: stem, loop, hairpin, spacer, linker, inverted repeat, cloning site ...

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US A 6054299 (CONRAD) 25 April 2000	1-41
A	WO A 98/18811 (CONRAD) 7 May 1998	1-41

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 2002	14 NOV 2002

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Christopher Luton Telephone No : (02) 6283 2256

NUC 39195

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01326

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 42-43

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Claims 42 and 43 do not define the matter for which protection is sought in terms of the technical features of the invention (Rule 6.3(a), Part B: Rules Concerning Chapter I of the Treaty).

3. ☐ Claims Nos :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU02/01326

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	6054299	NONE					
WO	9818811	AU	51595/98	EP	948513	US	5814500

END OF ANNEX

NUC 39197